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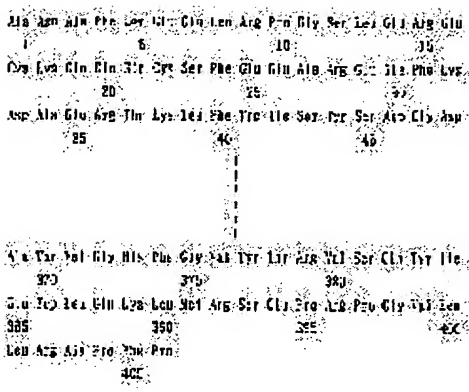
(71)Applicant : CHEMO SERO THERAPEUT RES INST

(72)Inventor : SOEJIMA KENJI
MIZUGUCHI JUN
YUGUCHI MASATO
NAKAGAKI TOSHIHIRO
IWANAGA SADAAKI

(54) **MODIFIED BLOOD COAGULATION FACTOR VII**

(57)Abstract:
PROBLEM TO BE SOLVED: To obtain a new (activated) modified blood coagulation factor VII which has a modification at a specific site of an amino acid sequence, has an enhanced enzyme activity, and is useful as a medicine effective for treating a hemophilia inhibitor patient or the like.
SOLUTION: This is a new (activated) modified blood coagulation factor VII (FVII) which has at least one modification(s) selected from a group comprising cleaving the disulfide bond (159Cys-164Cys) consisting of 159th cysteine (159Cys) and 164th cysteine (164Cys) in blood coagulation factor VII, substituting, adding, or deleting at least a part of the amino acid sequence constituting the loop structure consisting of the amino acid sequence from 233rd threonine (233Thr) to 240th asparagine (240Asp) in FVII, and substituting, adding, or deleting at least a part of the amino acid sequence constituting the intervening sequence consisting from 304th arginine (304Arg) to 329th cysteine (329Cys) in FVII, and has an enhanced enzyme activity.



CLAIMS

[Claim(s)]
[Claim 1] The alteration field of the blood coagulation factor VII (following, FVII) characterized by including at least one alteration chosen from the following, or activated type blood coagulation factor VII (following, FVIIa).
(a) Cut the disulfide bond (159Cys-164Cys) which consists of the 159th cysteine (159Cys) in FVII, and the 164th cysteine (164Cys).
(b) Replace, add or delete the amino acid sequence which constitutes the loop structure (99-loop may be called hereafter) which consists of an amino acid sequence of the 240th asparagine (240Asn) from the 233rd ********** (233Thr) in FVII, or its part.
(c) Replace, add or delete the amino acid sequence which constitutes the mediation amino acid sequence of the 329th cysteine (329Cys) from the 304th arginine (304Arg) in FVII, or its part.
[Claim 2] The alteration field according to claim 1 characterized by cutting the concerned 159Cys-164Cys by replacing the above-mentioned 159Cys and 164Cyses by amino acid residues other than Cys.
[Claim 3] The alteration field according to claim 1 or 2 which consists of an amino acid sequence of array table array number 4 publication.
[Claim 4] The alteration field according to claim 1 characterized by cutting 159Cys-164Cys and forming a disulfide bond (159Cys-299Cys) between 159Cyses and 299Cyses by amino acid residues other than Cys replacing 164Cyses, and replacing the 299th valine (299Val) by Cys.
[Claim 5] The alteration field according to claim 1 or 4 which consists of an amino acid sequence of array table array number 6 publication.
[Claim 6] The alteration field according to claim 1 characterized by being replaced by the amino acid sequence to which the amino acid sequence of 99-loop of FVII corresponds on the structure of other trypsin group serine proteases.
[Claim 7] The alteration field according to claim 1 or 6 other trypsin group serine proteases of whose are Homo-sapiens trypsins.
[Claim 8] The alteration field according to claim 1, 6, or 7 characterized by being replaced by Asp-Arg-Lys-Thr-Leu which has an amino acid sequence from the 235th valine in 99-loop of FVII (235Val) to the 239th ********** (239Thr) in the loop structure of corresponding on the structure of a Homo-

sapiens trypsin.
[Claim 9] The claim 1 which consists of an amino acid sequence of array table array number 8 publication, or the alteration field given in either 6-8.
[Claim 10] The alteration field according to claim 1 characterized by being replaced by the amino acid sequence to which the amino acid sequence which constitutes the mediation amino acid sequence of the 329th cysteine (329Cys) from the 304th arginine (304Arg) in FVII, or its part corresponds on the structure of other trypsin group serine proteases.
[Claim 11] The alteration field according to claim 1 or 10 other trypsin group serine proteases of whose are Homo-sapiens trypsins.
[Claim 12] The alteration field according to claim 1, 10, or 11 with which the amino acid sequence which constitutes the mediation amino acid sequence (170-loop may be called hereafter) of the 329th cysteine (329Cys) from the 310th cysteine (310Cys) in FVII, or its part is replaced, added or deleted.
[Claim 13] The claim 1 characterized by being replaced by Glu-Ala-Ser-Tyr-Pro-Gly-Lys which has an amino acid sequence from the 311st leucine in 170-loop of FVII (311Leu) to the 322nd asparagine (322Asn) in the loop structure of corresponding on the structure of a Homo-sapiens trypsin, or the alteration field given in either 10-12.
[Claim 14] The claim 1 which consists of an amino acid sequence of array table array number 10 publication, or the alteration field given in either 10-13.
[Claim 15] The amino acid sequence from the 235th valine in 99-loop of FVII (235Val) to the 239th ********** (239Thr) It is replaced by Asp-Arg-Lys-Thr-Leu in the loop structure of corresponding on the structure of a Homo-sapiens trypsin. And the amino acid sequence from the 311st leucine in 170-loop (311Leu) to the 322nd asparagine (322Asn) The alteration field according to claim 1 characterized by being replaced by Glu-Ala-Ser-Tyr-Pro-Gly-Lys in the loop structure of corresponding on the structure of a Homo-sapiens trypsin.
[Claim 16] The alteration field according to claim 1 or 15 which consists of an amino acid sequence of array table array number 12 publication.
[Claim 17] The drug constituent which contains the alteration field of a publication as an active principle in either of the claims 1-16.
[Claim 18] A medicine effective in the treatment of the hemophilia inhibitor patient who consists of a drug constituent of a claim 17.

DETAILED DESCRIPTION

[Detailed Description of the Invention]
[0001]
[The technical field to which invention belongs] The invention in this application relates to the alteration field of the blood coagulation factor VII (FVII may be called hereafter) which reinforced enzyme activity, and/or activation blood coagulation factor VII (FVIIa may be called hereafter). In detail, the invention in this application relates to a medicine effective in the treatment of the hemophilia inhibitor patient who consists of a drug constituent which contains the FVII/FVIIa alteration field with which activity was reinforced, and the concerned alteration field as an active principle, and the concerned drug constituent by replacing and suffering a loss in an amino acid sequence peculiar to FVII.
[0002]
[A proior art and the technical probrem which should be solved] FVII is the blood coagulation factor of a vitamin K dependency, and it is known widely that it is the initiation factor of external cause system blood coagulation. It has Gla field which becomes the amino acid sequence from an amino terminus to 35 residues from ten gamma carboxy glutamic acid (Gla may be called hereafter) like other vitamin K dependency coagulation factors (vol. Proc.Natl.Acad.Sci.USA, 83, p.2412-2416, 1986). FVII is set to in vitro. The activation blood coagulation Xth factor By (FXa may be called hereafter), activation blood coagulation factor IX (FIXa may be called hereafter), or the thrombin (FIIa may be called hereafter) Being changed, active FVII, i.e., FVIIa, which consists of an H chain by which 152Arg-153Ile was understood an added water part, and the bridge was constructed over it by the S-S bond of a piece, and an L chain, is known (J. Biol.Chem., vol. 251, p.4797-4802, 1976).
[0003] If the enzyme activity of the FVIIa [itself] is very weak and it combines with the tissue factor (TF) which is a coenzyme, it will go up dramatically (Komiyama et al., Biochemistry, 29 (40), and pp.9418-25 (1990)). Although the bonding site between biparite children is also made clear on amino-acid-residue level to the primary structure of FVIIa and TF, the crystal structure of the complex, and the pan, the detail (spacial-configuration change accompanied by TF combination) of the catalytic

activity multiplication mechanism is still unknown (Banner et al., et al., and Nature 380(6569):pp.41-6 (1996)).

[0004] As a replacement therapy to hemophilia A and a hemophilia B patient, medication of blood coagulation factor VIII (FVIII may be called hereafter) and a blood coagulation factor IX (FIX may be called hereafter) tablet is performed. However, in connection with the concerned cure, the occurrence of the neutralizing antibody (called an inhibitor) to FVIII and FIX is regarded as questionable.

[0005] As a symptomatic treatment of the hemophiliac who produced such an inhibitor, there are medication of the complex tablet which consists of medication of superfluous medication of (1) FVIII factor and a (2) swine FVIII factor, (3) FII, and FVII, FIX and FX, medication of (4) FVIIa tablet, etc. however, such technique -- respectively -- (1) -- more -- high -- about the shock according to an antigenicity about the symptom aggravation by lead of a potency inhibitor, and (2), and (3), the cost has [4 / (4) / a thrombus induction of DIC, and] problems, such as being high, by that a curative effect is inadequate, or extensive and a frequent administration When the balance of an effect and danger is taken into consideration in these, the most efficient thing is medication of FVIIa tablet of (4). However, in order to demonstrate the hemostasis effect for the weakness of the activity, FVIIa tablet needs extensive medication and a frequent administration, as mentioned above, and is raising the treatment cost greatly. Moreover, if compared with the conventional replacement therapy to which the curative effect is also performed to the hemophiliac, it cannot be said that it is enough.

[0006] Although producing the alteration field of FVII which raised enzyme activity as a means for solving this problem is mentioned, it is known that this is generally difficult (proteinic structure admission, Yukiteru Katsube editorial supervisions, educational company issue, 1992). It is considered by the ground [blood coagulation factor] of the following especially for the activity reinforcement by alteration to be difficult.

[0007] It is classified into two of the activity falls depended qualitatively unusually with the activity fall accompanied by a quantitative deficit although hemophilias are the abnormalities of a blood coagulation factor. Among these, masses [the example from which it becomes clear that the abnormalities in a molecule exist over the structure whole region of FIX, only one amino acid was only replaced by inside, and activity becomes 1% or less] as a result of knowing that many of qualitative abnormalities are mutations (point) and performing analysis of the patient of the hemophilia B which is the abnormalities of FIX. Therefore, even if it changes recklessly about a blood coagulation factor, it is clear to cause an activity fall.

[0008] According to the information (Dickinson et al., Proc.Natl.Acad.Sci.USA, 93 (25), and pp.14379-84 (1996)) acquired by Alanine Scannning, 112 Alanine substitution-product ******s of FVII and the thing which enzyme activity went up in it are only [one], and, moreover, the regularity is not found out.

[0009] As other attempts, Hopfner et al. produced FIX fragmentation alteration field which raised synthetic substrate activity using the technique of suffering for it a loss and replacing the structural unit which consists of a number amino acid residue of the domain of the part which constitutes FIX (EMBO J, 16 (22), and pp.6626-35 (1997)). However, since this made the intact partial fragmentation of not FIX but FIX discover by Escherichia coli and is looking at synthetic substrate activity, not to mention it can reinforce blood coagulation activity, it does not have even blood coagulation activity. Furthermore, this is not suggested at all to FVII which is the quality of a different thing from which structure and a specificity are completely different about FIX, and there is no report in any way until now also about the alteration field which reinforced the enzyme activity of FVII.

[0010] Thus, production of the alteration field which has strong enzyme activity was considered to be difficult especially in the blood coagulation factor. In FIX, although the attempt which raises synthetic substrate activity about the partial fragmentation was made, about the alteration field of the blood coagulation factor which has enzyme activity high as an intact molecule, there is no example of a report until now.

[0011] Therefore, the technical probrem which should solve this invention is producing and offering FVII and/or FVIIa which have strong activity effective in a hemophilia inhibitor patient's treatment in the status considered that an alteration of a blood coagulation factor is difficult generally.

[0012]

[Means for Solving the Problem] In the above statuses, this invention persons came to complete the invention in this application, as a result of repeating a research zealously that FVII which has enzyme activity high in itself should be produced and performing various studies. It succeeds in the invention in this application producing FVII and/or FVIIa alteration field with which activity was reinforced by comparing FVII and various serine proteases with amino-acid-sequence structure, clarifying an amino-acid-sequence site peculiar to FVII, and suffering for it a loss and replacing the characteristic site.

[0013]

[Elements of the Invention] a group similar to a trypsin group -- the basic structure of a serine protease consists of about 250 residues, and is about divided into two domains, the first half and the second half, on an amino acid sequence (drawing 1) Six beta strands are in each domain, respectively, and it is formed with the structure of having beta strand of a total of 12 as a protease (drawing 2). So to speak, these 12 beta strands have the skeletal structure of a serine protease, and the loop which connects between each strand, or the helix field is considered to bear protease activity, such as the substrate specificity and reactivity with a cofactor. As an example of a serine protease, there are digestive enzymes, such as thrombus lysis enzymes, such as various blood coagulation factors, such as FII, FVII, FVIII, FIX, and FX, and a plasmin, or a trypsin, a chymotrypsin, and an elastase. Then, amino-acid-sequence structure of the various serine proteases including FVII was compared, and the field characteristic of FVII was pinpointed (drawing 3). And it considered as the target of an alteration of these sites, and FVII alteration field which has high enzyme activity was produced by suffering for it a loss and replacing the amino acid sequence of FVII for the structure of other serine proteases by reference. These alteration fields are explained in detail.

[0014] (a) The alteration field with which the concerned 159Cys-164Cys was cut by replacing alteration field (a-**) 159Cys from which 159Cys-164Cys combination was cut, and 164Cyses by amino acid residues other than Cys (VII-5). As an example of this alteration field, what replaced Cys by the alanine (Ala), respectively was indicated for the array table array numbers 3 or 4. Here, as an example of amino acid residues other than Cys used for a substitute, although Ala was chosen, unless a serious failure of making enzyme activity deactivate besides cutting Cys-Cys combination etc. is done by the substitute, arbitrary amino acid is selectable.

[0015] (a-**) The alteration field with which 159Cys-164Cys was cut and the disulfide bond (159Cys-299Cys) was formed between 159Cyses and 299Cyses by amino acid residues other than Cys replacing 164Cys, and replacing the 299th ******** (299Val) by Cys (VII-6). What was replaced as an example of this alteration field, using Ala as amino acid residues other than Cys was indicated for the array table array numbers 5 or 6. Unless a serious failure of making enzyme activity deactivate besides cutting 159Cys-164Cys combination by the substitute etc. is done about an amino acid residue except Cys used for a substitute here as above-mentioned, other amino acid other than Ala is selectable.

[0016] (b) The alteration field with which the amino acid sequence which constitutes the loop structure (99-loop may be called hereafter) which consists of an amino acid sequence of the 240th asparagine (240Asn) from the 233rd ********** (233Thr) in FVII, or its part was replaced, added or deleted. This field contains the amino acid sequence which intervenes between the beta strand 5 which exists common to a serine protease as shown in drawing 3 , and the beta strand 6. It is desirable to replace this field by the amino acid sequence which corresponds on the structure of other trypsin group serine proteases. A Homo-sapiens trypsin is mentioned as a suitable example of a trypsin group serine protease. Furthermore, the alteration field (VII-30) with which the amino acid sequence from the 235th valine in 99-loop of FVII (235Val) to the 239th ********** (239Thr) was replaced by Asp-Arg-Lys-Thr-Leu in the loop structure of a trypsin as a concrete example is mentioned. This alteration field was indicated for the array table array numbers 7 or 8.

[0017] (c) The alteration field with which the amino acid sequence which constitutes the mediation amino acid sequence of the 329th cysteine (329Cys) from the 304th arginine (304Arg) in FVII, or its part was replaced, added or deleted.

the amino acid sequence which intervenes between the beta strand 8 which exists common to a serine protease, and the beta strand 9 as especially this field is shown in drawing 3 -- setting -- the serine protease of others [FVII] -- comparing -- a number amino acid residue -- since it has the characteristic feature of being long, what may serve as the suitable target in FVII alteration is conjectured It is desirable to replace this field by the amino acid sequence which corresponds on the structure of other trypsin group serine proteases. A Homo-sapiens trypsin is mentioned as a suitable example of a trypsin group serine protease. Moreover, the substitute in FVII and the desirable field which is added and can be deleted are the amino acid sequence which constitutes the loop structure (170-loop may be called) which consists of an amino acid sequence of the 329th cysteine (329Cys) from the 310th cysteine (310Cys), or its part. Furthermore, the alteration field (VII-31) with which the amino acid sequence from the 311st leucine in 170-loop of FVII (311Leu) to the 322nd asparagine (322Asn) was replaced by Glu-Ala-Ser-Tyr-Pro-Gly-Lys in the loop structure of a Homo-sapiens trypsin as a concrete example is mentioned. This alteration field was indicated for the array table array numbers 9 or 10.

[0018] Furthermore, it is also possible to combine an alteration of (c) suitably from the above (a). the example ****** -- for example, (b) and the combination of (c) -- that is The amino acid sequence from the 235th valine in 99-loop of FVII (235Val) to the 239th ********** (239Thr) It is replaced by Asp-Arg-Lys-Thr-Leu in the loop structure of a Homo-sapiens trypsin. And the amino acid sequence from the 311st leucine in 170-loop (311Leu) to the 322nd asparagine (322Asn) The alteration field (VII-39)

replaced by Glu-Ala-Ser-Tyr-Pro-Gly-Lys in the loop structure of a trypsin is mentioned. This alteration field was indicated for the array table array numbers 11 or 12.

[0019] The alteration field mentioned above can be acquired using the recombining [a gene] method. As a manifestation recipient, eukaryotic cells, such as an animal cell, are desirable. The alteration field of this invention includes cDNA which carries out the code of the amino acid sequence of each above-mentioned alteration field in a suitable manifestation vector, transfects a host cell, and is acquired by refining after carrying out the cloning of the cell which has discovered the target gene and cultivating the obtained stable manifestation stock.

[0020] FVII alteration field of the invention in this application can perform various chemical treatments etc., and can use them as activated type FVII (FVIIa) alteration field.

[0021] The FVII/FVIIa alteration field of the invention in this application can be prescribed to a pharmaceutical preparation because of the treatment, a diagnosis, or other intended use. To the preparation for intravenous administration, it melts into the aqueous solution which has buffered pH which may suit a physiological conditions in a constituent, including the matter which may usually suit physiologically, for example, a sodium chloride, a glycine, etc. Moreover, from the viewpoint of reservation of long term stability, as a final pharmaceutical form, taking the gestalt of a freeze-drying tablet is also taken into consideration, and it gets. In addition, the guideline of the constituent prescribed for the patient into the vena is established under the governmental rule, for example, "biological-preparation criteria." The treatment of the hemophilia inhibitor patient who produced the inhibitor to the concerned blood coagulation factor by the replacement therapy of FVIII or FIX as concrete intended use of the drug constituent which consists of the FVII/FVIIa alteration field of the invention in this application is mentioned.

[0022]

[Example] Although the invention in this application is illustrated according to an example, these examples do not limit the invention in this application. With reference to an accompanying drawying, it illustrates in the example [****] about the invention in this application. An example makes the alteration field discover in the culture supernatant of an animal cell (CHO-K1). The reagent in connection with [as long as there is no notice especially the following] transgenics etc. is TAKARA SHUZO, Toyobo, and par ********** applied New England Biolabs. The product of a shrine was used.

[0023] The cloning>> Homo-sapiens liver cDNA library (TAKARA SHUZO) of <<example 1.FVIIcDNA is purchased. Reference () etc. [Molecular Basis] of Thrombosia and cDNA array well-known at Hemostasis (-- FVII synthetic DNA sense primer (VII-PWN;GGGGTCGACATGGTCTCCCAGGCCCTCAGGCTCCTCTGCCTTCTG) which added SalI site to the array table array number 1 on the basis of written) -- and PCR is performed using the anti sense primer (VII-PWC;CCCGGATCCCTAGGGAAATGGGGCTCGCAGGAGGACTCCTGGGCG) which added BamHI site. The cloning was carried out to commercial cloning vector pCRII (Invitrogen). In this case, DNA sequence was performed by the conventional method and it checked having a well-known array (Hagen FS et al and PNAS 1986; 83; 2412-6) by reference etc.

[0024] Manufacture>> manifestation vector pCAGn (patent official report of No. 2824434) of a <<example 2.FVII manifestation vector was digested by SalI and BamHI, the ligation of what cut the DNA fragment prepared in the above-mentioned example 1 containing the array which carried out the code of the FVII there by SalI and BamHI was carried out, the transformation was carried out to Escherichia coli JM105, it cultivated on LB agar medium of ampicillin inclusion, and transformation Escherichia coli was chosen. It cultivated by the culture medium of marketing of the colony which appeared overnight, extraction refining of the target manifestation plasmid was carried out, and "pVII-W" was prepared. DNA sequence of this manifestation vector was performed and it checked having the target gene sequence.

[0025] Each FVII alteration field which has the amino acid sequence shown in manufacture>> view 4 of a <<example 3. alteration field manifestation vector was created by the following technique. In addition, drawing 4 is what showed only the amino acid sequence by the side of the end of C from the 153rd isoleucine of FVII, and about the amino acid by the side of the end of N, an alteration is not performed but is all the same than the 152nd arginine as that of a wild type. PCR is performed, using FVII gene as mold using the synthetic DNA primer shown in drawing 5 , and each amplification fragment is obtained. The ligation of each amplification fragment and the thing which cut manifestation vector pCAGn by SalI and BamHI was carried out, the transformation was carried out to Escherichia coli JM105, it cultivated on LB agar medium of ampicillin inclusion, and transformation Escherichia coli was chosen. It cultivated by the culture medium of marketing of the colony which appeared overnight, extraction refining of the target manifestation plasmid was carried out, and "pVII-5", "pVII-30", and "pVII-31" were prepared (drawing 6). Moreover, about "pVII-6", the gene obtained in

drawing 5 using primer ** and ** of a publication is used as mold, and it was obtained by performing PCR further using primer ** and **. Moreover, about "pVII-39", the gene obtained using primer ** and (10) is used as mold, and it was obtained by performing PCR further using a primer (11) and (12). Furthermore DNA sequence was performed and it checked that these plasmids had the target array.
[0026] The commercial ******** cutin reagent performed the transduction to CHO cell, the manifestation to the culture supernatant of <<example 4. each alteration field and the refining>> above-mentioned manifestation vector were chosen by G418 (1mg/(ml)), and the cloning of the cell which has discovered the target gene was carried out with the extra dilution method. ELISA kit (****** chromium FVII;Diagnostica Strago) to commercial FVII performed authentication of a manifestation of FVII alteration field. The obtained stable manifestation stock was cultivated by the serum free medium (ASF104, Ajinomoto, penicillin, streptomycin, 20microg [/ml] vitamin K, 1mM butyric acid), and was refined in the anti-Homo-sapiens FVII monoclonal antibody column (patent official report of No. 2824430). An equilibration, washing, and elution were performed using the equilibration and the washing buffer (50mM Tris, pH 7.2, 0.1M NaCl, 50mM Benzamidine-HCl, and 2mM calcium2+), and the elution buffer (50mM Tris, pH 7.2, 0.1MNaCl, 50mM Benzamidine-HCl, and 10mM EDTA). Using the antibody [as opposed to SDS-PAGE or commercial FVII for the purified alteration field], the Western blot was performed and it checked that it was FVII alteration field.
[0027] The freezing activity of measurement>> each alteration field of the freezing activity of <<example 5. each alteration field was measured by the solidifying method using FVII lack plasma according to the conventional method. each refined alteration field is set to 50 to 5 ng/ml -- as -- Tris-BSA -- diluting -- FVII lack ****, equivalent ****, and 37 degrees C -- 3 minutes -- warming -- equivalent addition of the formation TF (thromboplastin;Dade) of a re-lipid was carried out the back, and the freezing reaction was made to start The coagulation time was measured and it asked for freezing activity from the standard curve and the dilution ratio. The result which converted freezing activity into per [protein concentration (it measures by the Bradford method)], and asked for the specific activity is described in Table 1. Consequently, FVII alteration field of this invention became clear [having freezing activity high two to 6 times] as compared with plasma origin FVII and wild-type recombination FVII.
[0028]
[Table 1]

サンプル	改変内容	凝固活性 U/ml	蛋白濃度 μg/ml	比活性 U/ml	相対比 %
血漿由来	天然品	2,000	1,000	2,000	100
VII-W	組換え野生型	3,400	1,700	2,000	100
VII-5	159Cys-164Cys の切断	4,954	1,032	4,800	240
VII-6	159Cys-299Cys の形成	6,636	1,293	5,132	257
VII-30	loop99 を Trypsin 型へ	3,361	685	4,907	245
VII-31	loop170 を Trypsin 型へ	3,589	877	4,093	205
VII-39	loop99+170 を Trypsin 型へ	8,954	773	11,584	579

[0029] Each alteration field each alteration field of which the <<example 6. activation was done did whose manufacture>> refining is dialyzed to 50mM Tris, pH 7.45, and 0.1M NaCl. FXa is added 1/100 (mole ratio). 50mM Tris, pH 7.45, 0.1M NaCl, 0.1% PEG 8000, 100microg / mL phospholipid (Platerin (registered trademark) Organotecnica), and 10mM calcium2+, Under the 37-degree C condition, in 1 - 60 minutes, the incubation was carried out and it activated. After the activation, 50mM Benzamidine-HCl was added, the reaction was stopped, and it refined in the anti-Homo-sapiens FVII monoclonal antibody column (the same technique as an example 4). Each activation alteration field [finishing / refining] was dialyzed to TBS pH 8.0 (0.1% PEG 8000 inclusion), and carried out the cryopreservation to -80 degrees C. The grade of an activation was checked by SDS-PAGE.
[0030] Alteration field VIIa-31 activated according to the hydration activity-measurement>> example 6 over the synthetic substrate of each alteration field of which the <<example 7. activation was done until it is set to 0.1microM It dilutes with 50mMTris-HCl, 100mM NaCl, 10mM calcium2+, 0.1% PEG 8000, and pH 8.0. In addition, set the last capacity to 200microl, it was made to react at 30 degrees C so that various synthetic substrates may be set to last concentration 1.0mM there, and the amount of hydrations of the substrate per for 1 minute was seen. A temperature control is possible. Disengagement of pNA was measured as a degree of coloring by 405nm by microplate reader Spectra max plus (Molecular device). This result is shown in Table 2. VIIa-31 which are one of the alteration fields of this invention also received the synthetic substrate of what **, and showed hydration activity

higher than a wild type (VIIa-W), and the domain was two to 23 times.

[0031]

[Table 2]

基質名	構造	水解活性／mOD_{405nm}/min		比
		VIIa-W	VIIa-31	31/W
Chromozym tPA	D-Phe-Gly-Arg	37.6	117.6	3
S-2288	H-D-Ile-Pro-Arg	25.8	304.2	12
S-2366	pyro-Glu-Pro-Arg	11.5	267.8	23
S-2238	H-D-Phe-Pip-Arg	11.3	86.6	8
Chromozym X	D-Nile-Gly-Arg	13.0	48.6	4
S-2302	H-D-Pro-Phe-Arg	7.4	38.3	5
S-2765	Z-D-Arg-Gly-Arg	13.3	20.6	2
Chromozym TRY	CBz-Val-Gly-Arg	5.6	28.2	5
S-2444	pyro-Glu-Gly-Arg	1.5	18.9	13
S-2222	Bz-Ile-Glu-Gly-Arg	5.6	16.4	3
S-2403	pyro-Glu-Phe-Lys	0.3	5.9	19

[0032]

[Effect of the Invention] Thus, the alteration field of FVII obtained by the invention in this application and/or FVIIa has clearly high cnzyme activity compared with FVII of a wild type. Therefore, the alteration field of the invention in this application may serve as the very effective medicine as a replacement therapy to a hemophilia inhibitor patient.

[Layout Table]

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Gly Asp Ser 305 310 315 320 CCA AAT ATC ACG GAG TAC ATG TTC TGT GCC GGC TAC TCG GAT GGC AGC 1008Pro Asn Ile Thr Glu Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser 325 330 335 AAG-GAC-TCC-TGC-AAG GGG-GAC-AGT-GGA-GGC CCA CAT GCC ACC CAC TAC 1056Lys Asp Ser Cys Lys Gly Asp-Ser-Gly-Gly-Pro-His-Ala-Thr-His-Tyr 340 345 350 CGG-GGC ACG TGG TAC CTG ACG GGC ATC GTCAGC TGG GGC CAG GGC TGC 1104Arg Gly Thr Trp Tyr Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys 355 360 365 GCAACC GTG GGC CAC TTT GGG GTG TAC ACC AGG GTC TCC CAG TACATC 1152Ala Thr Val Gly His Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile 370 375 380 GAG TGG CTG CAAAAGCTC ATG CGC TCA GAG CCA CGC CCA GGA GTC CTC 1200Glu Trp Leu Gln Lys Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu 385 390 395 400 CTG CGA GCC CCA TTT CCC TAG 1221Leu Arg Ala Pro Phe Pro 405 <210> 2 <211> 406<212> PRT<213> blood coagulation factorVII<400> 2Ala Asn Ala Phe Leu Glu Glu Leu Arg Pro Gly Ser Leu Glu ArgGlu 1 5 10 15 Cys Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg GluIle 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Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp 245 250255 HisValVal Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr 260 265 270Leu Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu 275 280 285 AspArg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg 290 295 300Leu Met Thr Gln Asp Cys Leu Gln Gln Ser Arg Lys Val Gly Asp Ser 305 310 315320 Pro AsnIleThr Glu Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser 325 330 335Lys Asp Ser Cys Lys Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr 340 345 350Arg Gly Thr Trp Tyr Leu ThrGly IleVal Ser Trp Gly Gln Gly Cys 355 360 365AlaThr Val Gly His Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile 370 375 380Glu Trp Leu Gln Lys Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu 385 390 395400 Leu Arg Ala Pro Phe Pro 405 <210> 3<211> 1221<212> DNA <213> artificial sequence<220><223> Amino acid-sequence-of-recombinant mutant-of-blood-coagulation factor-VII-in-which both of the 159th Cysteine and the 164th Cysteine are-replaced-with-Alanine, and cDNA 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105 110 CGG TGC CAC GAG GGG TAC TCT CTG CTG GCA GACGGG GTG TCC TGC ACA 384Arg Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr 115 120 125 CCCACA GTT GAA TAT CCA TGT GGA AAA ATA CCT ATT CTA GAA AAA AGA432Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg 130 135 140 AATGCC AGC AAA CCC CAA GGC CGA ATT GTG GGG GGC AAG GTG GCC CCC 480Asn Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Ala Pro 145 150 155 160 AAA GGG GAG GCC CCA TGG CAG GTC CTG TTG TTGGTG AAT GGA GCT CAG 528Lys Gly Glu Ala Pro Trp Gln Val Leu Leu Leu Val Asn Gly Ala Gln 165 170 175 TTG-TGT-GGG-GGG-ACC CTG-ATC-AAC-ACC-ATC TGG GTG GTC TCC GCG GCC 576Leu Cys Gly Gly Thr Leu Ile-Asn-Thr-Ile-Trp-Val-Val-Ser-Ala-Ala 180 185 190 CAC-TGT-TTC-GAC-AAA ATC-AAG AAC TGG AGGAAC CTG ATC GCG GTG CTG 624His Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu 195 200 205 GGCGAG CAC GAC CTC AGC GAG CAC GAC GGG GAT GAG CAG AGC CGGCGG 672Gly Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg 210 215 220 GTG GCG CAG GTCATCATC CCC AGC ACG TAC GTC CCG GGC ACC ACC AAC 720Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn 225 230 235 240 CAC GAC ATC GCG CTG

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His Gln Pro Val Val Leu Thr Asp 245 250 255 CATGTG GTG CCC CTC TGC CTG CCC GAA CGG
ACG TTC TCT GAG AGG ACG816His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg
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ACC CACTAC 1056Lys Asp Ser Cys Lys Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr 340 345 350
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Thr Asn 225 230 235 240 His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp 245
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GCC CGG GAG ATC TTC AAG 96Cys Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe
Lys 20 25 30 GAC GCG GAG AGG ACG AAG CTG TTCTGG ATT TCT TAC AGT GAT GGG
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Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln 50 55 60 CTC CAG TCC TAT ATC TGC TTC
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Leu Leu Ala Asp Gly Val Ser Cys Thr 115 120 125 CCCACA GTT GAA TAT CCA TGT GGA AAA
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720Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn 225 230 235 240 CAC GAC
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Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn 225 230 235240 His AspIleAla Leu Leu Arg Leu His Gln
Pro Val ValLeu Thr Asp 245 250 255 His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg
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AAG-GAG-GAG-CAG TGC TCC TTC GAG GAG-GCC-CGG-GAG-ATC-TTC-AAG 96Cys Lys
Glu GluGln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys 20 25 30 GAC GCG GAG AGG ACGAAG
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Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg 130 135 140Asn Ala Ser LysPro Gln Gly Arg Ile ValGly Gly Lys Val Cys Pro 145 150 155 160 Lys Gly Glu Cys Pro Trp Gln Val Leu Leu Leu Val Asn Gly Ala Gln 165 170 175 Leu Cys Gly Gly Thr Leu-Ile-Asn-Thr-Ile Trp Val Val Ser Ala-Ala 180 185 190 His-Cys-Phe-Asp-Lys Ile Lys Asn Trp Arg-Asn-Leu-Ile-Ala-Val-Leu 195 200 205 Gly Glu His Asp Leu Ser Glu His Asp Gly AspGlu Gln Ser Arg Arg210215 220Val Ala Gln Val Ile Ile Pro Ser ThrTyr Asp Arg Lys Thr Leu Asn 225 230 235 240His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp 245 250 255 His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr 260 265270 LeuAla Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu 275 280 285AspArg Gly Ala Thr Ala Leu GluLeuMet Val LeuAsn Val Pro Arg 290 295 300 Leu Met Thr Gln Asp Cys Leu Gln Gln Ser Arg Lys Val Gly Asp Ser 305 310 315320 ProAsn Ile Thr Glu Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser 325 330335 LysAspSer Cys Lys Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr 340 345 350 Arg Gly Thr Trp Tyr Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys 355 360 365Ala Thr Val Gly His

Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile 370 375 380Glu Trp Leu GlnLys Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu 385 390 395 400Leu Arg Ala Pro Phe Pro 405 <210> 9 <211> 1206<212> DNA<213> artificial sequence<220> <223> Amino acid sequence of recombinant mutant of blood coagulation factor VII in which the 12 amino acid residues from the 311th leucine to 322th asparagine are replaced with Glu-Ala-Ser-Tyr-Pro-Gly-Lys and and cDNA sequence coding.[thereof] <400> 9GCC AAC GCG TTC CTG GAG GAG CTG CGG CCG GGC TCC CTG GAG AGG GAG 48Ala Asn Ala Phe Leu Glu Glu Leu Arg Pro-Gly-Ser-Leu-Glu Arg Glu 1 5 10 15 TGC-AAG-GAG-GAG-CAG TGC TCC TTC GAG GAG-GCC-CGG-GAG-ATC-TTC-AAG 96Cys Lys Glu Glu Gln-Cys-Ser-Phe Glu Glu Ala Arg Glu Ile Phe Lys 20 25 30 GAC GCG GAG AGG ACGAAG CTG TTC TGG ATT TCT TAC AGT GAT GGG GAC 144Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr SerAsp Gly Asp 35 40 45 CAG TGT GCC TCA AGTCCA TGC CAGAAT GGG GGC TCC TGC AAG GAC CAG 192Gln Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln 50 55 60 CTC CAG TCC TAT ATC TGC TTC TGC CTC CCT GCC TTC GAG GGC CGG AAC 240Leu Gln SerTyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn 65 70 75 80 TGTGAGACG CAC AAG GAT GAC CAG CTG ATC TGT GTG AAC GAG AAC GGC 288Cys Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly 85 90 95 GGC TGT GAG CAG TAC TGC AGT GAC CAC ACG GGC ACC AAGCGC TCCTGT 336Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys 100 105 110 CGG TGC CAC GAG GGG TAC TCT CTG CTGGCA GAC GGG GTG TCC TGC ACA 384Arg Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr 115 120 125 CCCACA GTT GAA TAT CCA TGT GGA AAA ATA CCT ATT CTA GAA AAAAGA 432Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg 130 135 140 AAT GCC AGC AAACCCCAA GGC CGA ATT GTG GGG GGC AAG GTG TGC CCC 480Asn Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Cys Pro 145 150 155 160 AAA GGG GAG TGT CCA TGG CAG GTC CTG TTG TTG GTG AAT GGA GCT CAG 528Lys Gly Glu Cys Pro Trp Gln Val Leu Leu Leu Val Asn Gly Ala 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Leu Leu Val Asn Gly Ala Gln 165 170175 TTGTGT GGG GGG ACC CTG ATC AAC ACC ATC TGG GTG GTC TCC GCG GCC 576Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala 180 185 190 CAC TGT TTC GAC AAA ATCAAGAAC TGG AGG AAC CTG ATC GCG GTG CTG 624His Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu 195 200 205 GGC-GAG-CAC-GAC-CTC AGC-GAG-CAC-GAC-GGG GAT GAG CAG AGC CGG CGG 672Gly Glu His Asp Leu Ser Glu-His-Asp-Gly-Asp-Glu-Gln-Ser-Arg-Arg 210 215 220 GTGGCG CAG GTC ATC ATC CCC AGC ACG TAC GACAGG AAG ACT CTG AAC 720Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Asp Arg Lys Thr Leu Asn 225 230 235 240CAC GAC ATC GCG CTG CTC CGC CTG CAC CAG CCC GTG GTC CTC ACT GAC 768His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp 245 250 255 CATGTG GTG CCC CTC TGC CTG CCC GAA CGG ACG TTC TCT GAG AGG ACG 816His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr 260 265 270 CTG GCC TTC GTG CGC TTC TCA TTG GTC AGC GGC TGG GGC CAG CTGCTG 864Leu Ala Phe Val Arg Phe Ser Leu Val Ser 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DETAILED DESCRIPTION

[Detailed Description of the Invention]

[0001]

[The technical field to which invention belongs] The invention in this application relates to the alteration field of the blood coagulation factor VII (FVII may be called hereafter) which reinforced enzyme activity, and/or activation blood coagulation factor VII (FVIIa may be called hereafter). In detail, the invention in this application relates to a medicine effective in the treatment of the hemophilia inhibitor patient who consists of a drug constituent which contains the FVII/FVIIa alteration field with which activity was reinforced, and the concerned alteration field as an active principle, and the concerned drug constituent by replacing and suffering a loss in an amino acid sequence peculiar to FVII.

[0002]

[A proior art and the technical probrem which should be solved] FVII is the blood coagulation factor of a vitamin K dependency, and it is known widely that it is the initiation factor of external cause system blood coagulation. It has Gla field which becomes the amino acid sequence from an amino terminus to 35 residues from ten gamma carboxy glutamic acid (Gla may be called hereafter) like other vitamin K dependency coagulation factors (vol. Proc.Natl.Acad.Sci.USA, 83, p.2412- 2416, 1986). FVII is set to in vitro. The activation blood coagulation Xth factor By (FXa may be called hereafter), activation blood coagulation factor IX (FIXa may be called hereafter), or the thrombin (FIIa may be called hereafter) Being changed, active FVII, i.e., FVIIa, which consists of an H chain by which 152Arg-153Ile was understood an added water part, and the bridge was constructed over it by the S-S bond of a piece, and an L chain, is known (J. Biol.Chem., vol. 251, p.4797- 4802, 1976).

[0003] If the enzyme activity of the FVIIa [itself] is very weak and it combines with the tissue factor (TF) which is a coenzyme, it will go up dramatically (Komiyama et al., Biochemistry, 29 (40), and pp.9418-25 (1990)). Although the bonding site between biparite children is also made clear on amino-acid-residue level to the primary structure of FVIIa and TF, the crystal structure of the complex, and the pan, the detail (spacial-configuration change accompanied by TF combination) of the catalytic activity multiplication mechanism is still unknown (Banner et al., et al., and Nature 380(6569):pp.41-6 (1996)).

[0004] As a replacement therapy to hemophilia A and a hemophilia B patient, medication of blood coagulation factor VIII (FVIII may be called hereafter) and a blood coagulation factor IX (FIX may be called hereafter) tablet is performed. However, in connection with the concerned cure, the occurrence of the neutralizing antibody (called an inhibitor) to FVIII and FIX is regarded as questionable.

[0005] As a symptomatic treatment of the hemophiliac who produced such an inhibitor, there are medication of the complex tablet which consists of medication of superfluous medication of (1) FVIII factor and a (2) swine FVIII factor, (3) FII, and FVII, FIX and FX, medication of (4) FVIIa tablet, etc. however, such technique -- respectively -- (1) -- more -- high -- about the shock according to an antigenicity about the symptom aggravation by lead of a potency inhibitor, and (2), and (3), the cost has [4 / (4) / a thrombus induction of DIC, and] problems, such as being high, by that a curative effect is inadequate, or extensive and a frequent administration When the balance of an effect and danger is taken into consideration in these, the most efficient thing is medication of FVIIa tablet of (4). However, in order to demonstrate the hemostasis effect for the weakness of the activity, FVIIa tablet needs extensive medication and a frequent administration, as mentioned above, and is raising the treatment cost greatly. Moreover, if compared with the conventional replacement therapy to which the curative effect is also performed to the hemophiliac, it cannot be said that it is enough.
[0006] Although producing the alteration field of FVII which raised enzyme activity as a means for solving this problem is mentioned, it is known that this is generally difficult (proteinic structure admission, Yukiteru Katsube editorial supervisions, educational company issue, 1992). It is considered by the ground [blood coagulation factor] of the following especially for the activity reinforcement by alteration to be difficult.
[0007] It is classified into two of the activity falls depended qualitatively unusually with the activity fall accompanied by a quantitative deficit although hemophilias are the abnormalities of a blood coagulation factor. Among these, masses [the example from which it becomes clear that the abnormalities in a molecule exist over the structure whole region of FIX, only one amino acid was only replaced by inside, and activity becomes 1% or less] as a result of knowing that many of qualitative abnormalities are mutations (point) and performing analysis of the patient of the hemophilia B which is the abnormalities of FIX. Therefore, even if it changes recklessly about a blood coagulation factor, it is clear to cause an activity fall.
[0008] According to the information (Dickinson et al., Proc.Natl.Acad.Sci.USA, 93 (25), and pp.14379-84 (1996)) acquired by Alanine Scannning, 112 Alanine substitution-product ******s of FVII and the thing which enzyme activity went up in it are only [one], and, moreover, the regularity is not found out.
[0009] As other attempts, Hopfner et al. produced FIX fragmentation alteration field which raised synthetic substrate activity using the technique of suffering for it a loss and replacing the structural unit which consists of a number amino acid residue of the domain of the part which constitutes FIX (EMBO J, 16 (22), and pp.6626-35 (1997)). However, since this made the intact partial fragmentation of not FIX but FIX discover by Escherichia coli and is looking at synthetic substrate activity, not to mention it can reinforce blood coagulation activity, it does not have even blood coagulation activity. Furthermore, this is not suggested at all to FVII which is the quality of a different thing from which structure and a specificity are completely different about FIX, and there is no report in any way until now also about the alteration field which reinforced the enzyme activity of FVII.
[0010] Thus, production of the alteration field which has strong enzyme activity was considered to be difficult especially in the blood coagulation factor. In FIX, although the attempt which raises synthetic substrate activity about the partial fragmentation was made, about the alteration field of the blood coagulation factor which has enzyme activity high as an intact molecule, there is no example of a report until now.
[0011] Therefore, the technical probrem which should solve this invention is producing and offering FVII and/or FVIIa which have strong activity effective in a hemophilia inhibitor patient's treatment in the status considered that an alteration of a blood coagulation factor is difficult generally.
[0012]
[Means for Solving the Problem] In the above statuses, this invention persons came to complete the invention in this application, as a result of repeating a research zealously that FVII which has enzyme activity high in itself should be produced and performing various studies. It succeeds in the invention in this application producing FVII and/or FVIIa alteration field with which activity was reinforced by comparing FVII and various serine proteases with amino-acid-sequence structure, clarifying an amino-acid-sequence site peculiar to FVII, and suffering for it a loss and replacing the characteristic site.
[0013]
[Elements of the Invention] a group similar to a trypsin group -- the basic structure of a serine protease consists of about 250 residues, and is about divided into two domains, the first half and the second half, on an amino acid sequence (drawing 1) Six beta strands are in each domain, respectively, and it is formed with the structure of having beta strand of a total of 12 as a protease (drawing 2). So to speak, these 12 beta strands have the skeletal structure of a serine protease, and the loop which connects between each strand, or the helix field is considered to bear protease activity, such as the substrate

specificity and reactivity with a cofactor. As an example of a serine protease, there are digestive enzymes, such as thrombus lysis enzymes, such as various blood coagulation factors, such as FII, FVII, FVIII, FIX, and FX, and a plasmin, or a trypsin, a chymotrypsin, and an elastase. Then, amino-acid-sequence structure of the various serine proteases including FVII was compared, and the field characteristic of FVII was pinpointed (drawing 3). And it considered as the target of an alteration of these sites, and FVII alteration field which has high enzyme activity was produced by suffering for it a loss and replacing the amino acid sequence of FVII for the structure of other serine proteases by reference. These alteration fields are explained in detail.

[0014] (a) The alteration field with which the concerned 159Cys-164Cys was cut by replacing alteration field (a-**) 159Cys from which 159Cys-164Cys combination was cut, and 164Cyses by amino acid residues other than Cys (VII-5). As an example of this alteration field, what replaced Cys by the alanine (Ala), respectively was indicated for the array table array numbers 3 or 4. Here, as an example of amino acid residues other than Cys used for a substitute, although Ala was chosen, unless a serious failure of making enzyme activity deactivate besides cutting Cys-Cys combination etc. is done by the substitute, arbitrary amino acid is selectable.

[0015] (a-**) The alteration field with which 159Cys-164Cys was cut and the disulfide bond (159Cys-299Cys) was formed between 159Cyses and 299Cyses by amino acid residues other than Cys replacing 164Cys, and replacing the 299th ******** (299Val) by Cys (VII-6). What was replaced as an example of this alteration field, using Ala as amino acid residues other than Cys was indicated for the array table array numbers 5 or 6. Unless a serious failure of making enzyme activity deactivate besides cutting 159Cys-164Cys combination by the substitute etc. is done about an amino acid residue except Cys used for a substitute here as above-mentioned, other amino acid other than Ala is selectable.

[0016] (b) The alteration field with which the amino acid sequence which constitutes the loop structure (99-loop may be called hereafter) which consists of an amino acid sequence of the 240th asparagine (240Asn) from the 233rd ********** (233Thr) in FVII, or its part was replaced, added or deleted. This field contains the amino acid sequence which intervenes between the beta strand 5 which exists common to a serine protease as shown in drawing 3 , and the beta strand 6. It is desirable to replace this field by the amino acid sequence which corresponds on the structure of other trypsin group serine proteases. A Homo -sapiens trypsin is mentioned as a suitable example of a trypsin group serine protease. Furthermore, the alteration field (VII-30) with which the amino acid sequence from the 235th valine in 99-loop of FVII (235Val) to the 239th ********** (239Thr) was replaced by Asp-Arg-Lys-Thr-Leu in the loop structure of a trypsin as a concrete example is mentioned. This alteration field was indicated for the array table array numbers 7 or 8.

[0017] (c) The alteration field with which the amino acid sequence which constitutes the mediation amino acid sequence of the 329th cysteine (329Cys) from the 304th arginine (304Arg) in FVII, or its part was replaced, added or deleted.

the amino acid sequence which intervenes between the beta strand 8 which exists common to a serine protease, and the beta strand 9 as especially this field is shown in drawing 3 -- setting -- the serine protease of others [FVII] -- comparing -- a number amino acid residue -- since it has the characteristic feature of being long, what may serve as the suitable target in FVII alteration is conjectured It is desirable to replace this field by the amino acid sequence which corresponds on the structure of other trypsin group serine proteases. A Homo -sapiens trypsin is mentioned as a suitable example of a trypsin group serine protease. Moreover, the substitute in FVII and the desirable field which is added and can be deleted are the amino acid sequence which constitutes the loop structure (170-loop may be called) which consists of an amino acid sequence of the 329th cysteine (329Cys) from the 310th cysteine (310Cys), or its part. Furthermore, the alteration field (VII-31) with which the amino acid sequence from the 311st leucine in 170-loop of FVII (311Leu) to the 322nd asparagine (322Asn) was replaced by Glu-Ala-Ser-Tyr-Pro-Gly-Lys in the loop structure of a Homo -sapiens trypsin as a concrete example is mentioned. This alteration field was indicated for the array table array numbers 9 or 10.

[0018] Furthermore, it is also possible to combine an alteration of (c) suitably from the above (a). the example ****** -- for example, (b) and the combination of (c) -- that is The amino acid sequence from the 235th valine in 99-loop of FVII (235Val) to the 239th ********** (239Thr) It is replaced by Asp-Arg-Lys-Thr-Leu in the loop structure of a Homo -sapiens trypsin. And the amino acid sequence from the 311st leucine in 170-loop (311Leu) to the 322nd asparagine (322Asn) The alteration field (VII-39) replaced by Glu-Ala-Ser-Tyr-Pro-Gly-Lys in the loop structure of a trypsin is mentioned. This alteration field was indicated for the array table array numbers 11 or 12.

[0019] The alteration field mentioned above can be acquired using the recombining [a gene] method. As a manifestation recipient, eukaryotic cells, such as an animal cell, are desirable. The alteration field of this invention includes cDNA which carries out the code of the amino acid sequence of each above-mentioned alteration field in a suitable manifestation vector, transfects a host cell, and is acquired by

refining after carrying out the cloning of the cell which has discovered the target gene and cultivating the obtained stable manifestation stock.
[0020] FVII alteration field of the invention in this application can perform various chemical treatments etc., and can use them as activated type FVII (FVIIa) alteration field.
[0021] The FVII/FVIIa alteration field of the invention in this application can be prescribed to a pharmaceutical preparation because of the treatment, a diagnosis, or other intended use. To the preparation for intravenous administration, it melts into the aqueous solution which has buffered pH which may suit a physiological conditions in a constituent, including the matter which may usually suit physiologically, for example, a sodium chloride, a glycine, etc. Moreover, from the viewpoint of reservation of long term stability, as a final pharmaceutical form, taking the gestalt of a freeze-drying tablet is also taken into consideration, and it gets. In addition, the guideline of the constituent prescribed for the patient into the vena is established under the governmental rule, for example, "biological-preparation criteria." The treatment of the hemophilia inhibitor patient who produced the inhibitor to the concerned blood coagulation factor by the replacement therapy of FVIII or FIX as concrete intended use of the drug constituent which consists of the FVII/FVIIa alteration field of the invention in this application is mentioned.
[0022]
[Example] Although the invention in this application is illustrated according to an example, these examples do not limit the invention in this application. With reference to an accompanying drawying, it illustratcs in the example [****] about the invention in this application. An example makes the alteration field discover in the culture supernatant of an animal cell (CHO-K1). The reagent in connection with [as long as there is no notice especially the following] transgenics etc. is TAKARA SHUZO, Toyobo, and par ********** applied New England Biolabs. The product of a shrine was used.
[0023] The cloning>> Homo-sapiens liver cDNA library (TAKARA SHUZO) of <<example 1.FVIIcDNA is purchased. Reference () etc. [Molecular Basis] of Thrombosis and cDNA array well-known at Hemostasis (-- FVII synthetic DNA sense primer (VII-PWN;GGGGTCGACATGGTCTCCCAGGCCCTCAGGCTCCTCTGCCTTCTG) which added SalI site to the array table array number 1 on the basis of written) -- and PCR is performed using the anti sense primer (VII-PWC;CCCGGATCCCTAGGGAAATGGGGCTCGCAGGAGGACTCCTGGGCG) which added BamHI site. The cloning was carried out to commercial cloning vector pCRII (Invitrogen). In this case, DNA sequence was performed by the conventional method and it checked having a well-known array (Hagen FS et al and PNAS 1986; 83; 2412-6) by reference etc.
[0024] Manufacture>> manifestation vector pCAGn (patent official report of No. 2824434) of a <<example 2.FVII manifestation vector was digested by SalI and BamHI, the ligation of what cut the DNA fragment prepared in the above-mentioned example 1 containing the array which carried out the code of the FVII there by SalI and BamHI was carried out, the transformation was carried out to Escherichia coli JM105, it cultivated on LB agar medium of ampicillin inclusion, and transformation Escherichia coli was chosen. It cultivated by the culture medium of marketing of the colony which appeared overnight, extraction refining of the target manifestation plasmid was carried out, and "pVII-W" was prepared. DNA sequence of this manifestation vector was performed and it checked having the target gene sequence.
[0025] Each FVII alteration field which has the amino acid sequence shown in manufacture>> view 4 of a <<example 3. alteration field manifestation vector was created by the following technique. In addition, drawing 4 is what showed only the amino acid sequence by the side of the end of C from the 153rd isoleucine of FVII, and about the amino acid by the side of the end of N, an alteration is not performed but is all the same than the 152nd arginine as that of a wild type. PCR is performed, using FVII gene as mold using the synthetic DNA primer shown in drawing 5 , and each amplification fragment is obtained. The ligation of each amplification fragment and the thing which cut manifestation vector pCAGn by SalI and BamHI was carried out, the transformation was carried out to Escherichia coli JM105, it cultivated on LB agar medium of ampicillin inclusion, and transformation Escherichia coli was chosen. It cultivated by the culture medium of marketing of the colony which appeared overnight, extraction refining of the target manifestation plasmid was carried out, and "pVII-5", "pVII-30", and "pVII-31" were prepared (drawing 6). Moreover, about "pVII-6", the gene obtained in drawing 5 using primer ** and ** of a publication is used as mold, and it was obtained by performing PCR further using primer ** and **. Moreover, about "pVII-39", the gene obtained using primer ** and (10) is used as mold, and it was obtained by performing PCR further using a primer (11) and (12). Furthermore DNA sequence was performed and it checked that these plasmids had the target array.
[0026] The commercial ******** cutin reagent performed the transduction to CHO cell, the manifestation to the culture supernatant of <<example 4. each alteration field and the refining>>

above-mentioned manifestation vector were chosen by G418 (1mg/(ml)), and the cloning of the cell which has discovered the target gene was carried out with the extra dilution method. ELISA kit (****** chromium FVII;Diagnostica Strago) to commercial FVII performed authentication of a manifestation of FVII alteration field. The obtained stable manifestation stock was cultivated by the serum free medium (ASF104, Ajinomoto, penicillin, streptomycin, 20microg [/ml] vitamin K, 1mM butyric acid), and was refined in the anti-Homo-sapiens FVII monoclonal antibody column (patent official report of No. 2824430). An equilibration, washing, and elution were performed using the equilibration and the washing buffer (50mM Tris, pH 7.2, 0.1M NaCl, 50mM Benzamidine-HCl, and 2mM calcium2+), and the elution buffer (50mM Tris, pH 7.2, 0.1MNaCl, 50mM Benzamidine-HCl, and 10mM EDTA). Using the antibody [as opposed to SDS-PAGE or commercial FVII for the purified alteration field], the Western blot was performed and it checked that it was FVII alteration field.

[0027] The freezing activity of measurement>> each alteration field of the freezing activity of <<example 5. each alteration field was measured by the solidifying method using FVII lack plasma according to the conventional method. each refined alteration field is set to 50 to 5 ng/ml -- as -- Tris-BSA -- diluting -- FVII lack ****, equivalent ****, and 37 degrees C -- 3 minutes -- warming -- equivalent addition of the formation TF (thromboplastin;Dade) of a re-lipid was carried out the back, and the freezing reaction was made to start The coagulation time was measured and it asked for freezing activity from the standard curve and the dilution ratio. The result which converted freezing activity into per [protein concentration (it measures by the Bradford method)], and asked for the specific activity is described in Table 1. Consequently, FVII alteration field of this invention became clear [having freezing activity high two to 6 times] as compared with plasma origin FVII and wild-type recombination FVII.

[0028]

[Table 1]

サンプル	改変内容	凝固活性 U/ml	蛋白濃度 μg/ml	比活性 U/ml	相対比 %
血漿由来	天然品	2,000	1,000	2,000	100
VII-W	組換え野生型	3,400	1,700	2,000	100
VII-5	159Cys-164Cys の切断	4,954	1,032	4,800	240
VII-6	159Cys-299Cys の形成	6,636	1,293	5,132	257
VII-30	loop99 を Trypsin 型へ	3,361	685	4,907	245
VII-31	loop170 を Trypsin 型へ	3,589	877	4,093	205
VII-39	loop99+170 を Trypsin 型へ	8,954	773	11,584	579

[0029] Each alteration field each alteration field of which the <<example 6. activation was done did whose manufacture>> refining is dialyzed to 50mM Tris, pH 7.45, and 0.1M NaCl. FXa is added 1/100 (mole ratio). 50mM Tris, pH 7.45, 0.1M NaCl, 0.1% PEG 8000, 100microg / mL phospholipid (Platerin (registered trademark) Organotecnica), and 10mM calcium2+, Under the 37-degree C condition, in 1 - 60 minutes, the incubation was carried out and it activated. After the activation, 50mM Benzamidine-HCl was added, the reaction was stopped, and it refined in the anti-Homo-sapiens FVII monoclonal antibody column (the same technique as an example 4). Each activation alteration field [finishing / refining] was dialyzed to TBS pH 8.0 (0.1% PEG 8000 inclusion), and carried out the cryopreservation to -80 degrees C. The grade of an activation was checked by SDS-PAGE.

[0030] Alteration field VIIa-31 activated according to the hydration activity-measurement>> example 6 over the synthetic substrate of each alteration field of which the <<example 7. activation was done until it is set to 0.1microM It dilutes with 50mMTris-HCl, 100mM NaCl, 10mM calcium2+, 0.1% PEG 8000, and pH 8.0. In addition, set the last capacity to 200microl, it was made to react at 30 degrees C so that various synthetic substrates may be set to last concentration 1.0mM there, and the amount of hydrations of the substrate per for 1 minute was seen. A temperature control is possible. Disengagement of pNA was measured as a degree of coloring by 405nm by microplate reader Spectra max plus (Molecular device). This result is shown in Table 2. VIIa-31 which are one of the alteration fields of this invention also received the synthetic substrate of what **, and showed hydration activity higher than a wild type (VIIa-W), and the domain was two to 23 times.

[0031]

[Table 2]

基質名	構造	水解活性／mOD$_{405nm}$/min		比
		VIIa-W	VIIa-31	31/W
Chromozym tPA	D-Phe-Gly-Arg	37.6	117.6	3
S-2288	H-D-Ile-Pro-Arg	25.8	304.2	12
S-2366	pyro-Glu-Pro-Arg	11.5	267.8	23
S-2238	H-D-Phe-Pip-Arg	11.3	86.6	8
Chromozym X	D-Nile-Gly-Arg	13.0	48.6	4
S-2302	H-D-Pro-Phe-Arg	7.4	38.3	5
S-2765	Z-D-Arg-Gly-Arg	13.3	20.6	2
Chromozym TRY	CBz-Val-Gly-Arg	5.6	28.2	5
S-2444	pyro-Glu-Gly-Arg	1.5	18.9	13
S-2222	Bz-Ile-Glu-Gly-Arg	5.6	16.4	3
S-2403	pyro-Glu-Phe-Lys	0.3	5.9	19

[0032]

[Effect of the Invention] Thus, the alteration field of FVII obtained by the invention in this application and/or FVIIa has clearly high enzyme activity compared with FVII of a wild type. Therefore, the alteration field of the invention in this application may serve as the very effective medicine as a replacement therapy to a hemophilia inhibitor patient.

[Layout Table]

SEQUENCE-LISTING<110> The-Chemo-Sero-Therapeutic Research-Institute<120> Recombinant-mutants of-blood-coagulation factor VII<160> 12<210> 1<211> 1221<212> DNA<213> blood coagulation factor VII<400> 1GCC AAC GCG TTC CTG GAG GAG CTG CGG CCG GGC-TCC-CTG GAG AGG GAG 48Ala Asn Ala Phe Leu Glu Glu Leu Arg Pro Gly Ser Leu Glu Arg Glu 15 1015 TGC AAG GAGGAG CAG TGC TCC TTC GAG GAG GCC CGG GAG ATC TTC AAG 96Cys Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys 20 25 30 GAC GCG GAG AGG ACG AAG CTG TTC TGG ATTTCT TAC AGT GAT GGG GAC 144AspAla Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp 35 40 45 CAG TGT GCC TCA AGT CCA TGC CAG AAT GGG GGC TCC TGC AAG GAC CAG 192Gln Cys Ala Ser SerPro Cys Gln Asn Gly GlySer Cys Lys Asp Gln 50 55 60 CTC CAG TCC TAT ATC TGC TTC TGC CTC CCT GCC TTC GAG GGC CGG AAC 240Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn 65 70 75 80TGT GAG ACG CAC AAG GAT GAC CAG CTG ATC TGT GTG AAC GAG AAC GGC288Cys Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly 85 90 95 GGC TGT GAG CAG TAC TGC AGT GAC CAC ACGGGC ACC AAG CGC TCC TGT 336Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys 100 105 110 CGG TGC CAC GAG GGG TAC TCT CTG CTG GCA GAC GGG GTG TCC TGC ACA 384Arg Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr 115 120 125 CCCACA GTT GAA TAT CCA TGT GGA AAAATA CCT ATT CTAGAA AAA AGA 432Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg 130 135 140 AATGCC AGC AAA CCC CAA GGC CGA ATT GTG GGG GGC AAG GTG TGC CCC 480Asn Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Cys Pro 145 150 155160 AAA GGG GAG TGT CCA TGG CAG GTC CTG TTG TTG GTG AAT GGA GCT CAG 528Lys Gly Glu Cys Pro Trp Gln Val Leu Leu Leu Val Asn Gly Ala Gln 165 170 175 TTG-TGT-GGG-GGG-ACC CTG-ATC-AAC-ACC-ATC TGG GTG GTC TCC GCG GCC 576Leu Cys Gly Gly Thr Leu Ile-Asn-Thr-Ile-Trp-Val-Val-Ser-Ala-Ala 180 185 190 CAC-TGT-TTC-GAC-AAA ATC-AAG-AAC-TGG-AGGAAC CTG ATC GCG GTG CTG 624His Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu 195 200 205 GGCGAG CAC GAC CTC AGC GAG CAC GAC GGG GAT GAG CAG AGC CGGCGG 672Gly Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg 210 215 220 GTG GCG CAG GTCATCATC CCC AGC ACG TAC GTC CCG GGC ACC ACC AAC 720Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn 225 230 235 240 CAC GAC ATC GCG CTG CTC CGC CTG CAC CAG CCC GTG GTC CTC ACT GAC 768His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp 245 250 255 CATGTG GTG CCC CTC TGC CTG CCC GAA CGG ACG TTC TCT GAG AGG ACG816His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr 260 265 270 CTG GCC TTC GTG CGC TTCTCATTG GTC AGC GGC TGG GGC CAG CTG CTG 864Leu Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu 275 280 285 GACCGT GGC GCC ACG GCC CTG GAG CTC ATG GTG CTC AAC GTG CCC CGG 912Asp Arg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg 290 295 300 CTGATG ACC CAG GAC TGC CTG CAG CAG TCA CGG AAG GTG GGA GAC TCC 960Leu Met Thr Gln Asp Cys Leu Gln Gln Ser Arg Lys Val Gly Asp Ser 305 310 315 320 CCA AAT ATC ACG GAG TAC ATG TTC TGT GCC GGC TAC TCG GAT GGC AGC 1008Pro Asn Ile Thr Glu Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser 325 330 335 AAG-GAC-TCC-TGC-AAG GGG-GAC-AGT-GGA-GGC CCA CAT GCC ACC CAC TAC

1056Lys Asp Ser Cys Lys Gly Asp-Ser-Gly-Gly-Pro-His-Ala-Thr-His-Tyr 340 345 350 CGG-GGC ACG TGG TAC CTG ACG GGC ATC GTCAGC TGG GGC CAG GGC TGC 1104Arg Gly Thr Trp Tyr Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys 355 360 365 GCAACC GTG GGC CAC TTT GGG GTG TAC ACC AGG GTC TCC CAG TACATC 1152Ala Thr Val Gly His Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile 370 375 380 GAG TGG CTG CAAAAGCTC ATG CGC TCA GAG CCA CGC CCA GGA GTC CTC 1200Glu Trp Leu Gln Lys Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu 385 390 395 400 CTG CGA GCC CCA TTT CCC TAG 1221Leu Arg Ala Pro Phe Pro 405 <210> 2 <211> 406<212> PRT<213> blood coagulation factorVII<400> 2Ala Asn Ala Phe Leu Glu Glu Leu Arg Pro Gly Ser Leu Glu ArgGlu 1 5 10 15 Cys Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg GluIle Phe Lys 20 25 30 Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp 35 4045 Gln Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln 50 5560 Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn 65 70 75 80Cys Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly 85 90 95Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys 100 105 110 Arg Cys HisGlu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr 115 120 125 Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg 130 135 140Asn AlaSerLys Pro Gln Gly Arg IleVal Gly Gly Lys Val Cys Pro 145 150 155 160 Lys-Gly-Glu-Cys-Pro Trp Gln Val Leu Leu-Leu-Val-Asn-Gly-Ala-Gln 165 170 175 Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser AlaAla180 185190 HisCys PheAsp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu 195 200205Gly Glu His Asp Leu Ser Glu His Asp Gly Asp Glu GlnSer Arg Arg 210 215 220Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn 225 230 235 240His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp 245 250255 His ValVal Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr 260 265 270Leu Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu 275 280 285 AspArg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg 290 295 300Leu Met Thr Gln Asp Cys Leu Gln Gln Ser Arg Lys Val Gly Asp Ser 305 310 315320 Pro AsnIleThr Glu Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser 325 330 335Lys Asp Ser Cys Lys Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr 340 345 350Arg Gly Thr Trp Tyr Leu ThrGly IleVal Ser Trp Gly Gln Gly Cys 355 360 365AlaThr Val Gly His Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile 370 375 380Glu Trp Leu Gln Lys Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu 385 390 395400 Leu Arg Ala Pro Phe Pro 405 <210> 3<211> 1221<212> DNA <213> artificial sequence<220><223> Amino acid-sequence-of-recombinant mutant-of-blood-coagulation factor-VII-in-which both of the 159th Cysteine and the 164th Cysteine are-replaced-with-Alanine, and cDNA sequence coding thereof.<400> 3GCC AAC GCG TTC CTG-GAG-GAG-CTG CGG CCG GGC TCC CTG GAGAGG GAG 48Ala Asn Ala PheLeu Glu Glu Leu Arg Pro Gly Ser Leu Glu Arg Glu 1 5 10 15 TGC AAG GAG GAG CAG TGC TCCTTC GAG GAG GCC CGG GAG ATC TTC AAG 96Cys Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys 20 25 30 GAC GCG GAG AGG ACG AAG CTG TTC TGGATT TCT TAC AGT GAT GGG GAC 144Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr SerAsp Gly Asp 35 40 45 CAG TGT GCC TCA AGT CCA TGC CAG AAT GGG GGC TCC TGC AAGGAC CAG 192Gln Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln 50 55 60CTC CAG TCC TAT ATC TGC TTC TGC CTC CCT GCC TTC GAG GGC CGG AAC240Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn 65 70 75 80 TGTGAG ACG CAC AAG GAT GAC CAG CTG ATC TGT GTG AAC GAG AAC GGC 288Cys Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly 85 90 95GGC TGT GAG CAG TAC TGC AGT GAC CAC ACG GGC ACC AAG CGC TCC TGT 336Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys 100 105 110 CGG TGC CAC GAG GGG TAC TCT CTG CTG GCA GACGGG GTG TCC TGC ACA 384Arg Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr 115 120 125 CCCACA GTT GAA TAT CCA TGT GGA AAA ATA CCT ATT CTA GAA AAA AGA432Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg 130 135 140 AATGCC AGC AAA CCC CAA GGC CGA ATT GTG GGG GGC AAG GTG GCC CCC 480Asn Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Ala Pro 145 150 155 160 AAA GGG GAG GCC CCA TGG CAG GTC CTG TTG TTGGTG AAT GGA GCT CAG 528Lys Gly Glu Ala Pro Trp Gln Val Leu Leu Leu Val Asn Gly Ala Gln 165 170 175 TTG-TGT-GGG-GGG-ACC CTG-ATC-AAC-ACC-ATC TGG GTG GTC TCC GCG GCC 576Leu Cys Gly Gly Thr Leu Ile-Asn-Thr-Ile-Trp-Val-Val-Ser-Ala-Ala 180 185 190 CAC-TGT-TTC-GAC-AAA ATC-AAG AAC TGG AGGAAC CTG ATC GCG GTG CTG 624His Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu 195 200 205 GGCGAG CAC GAC CTC AGC GAG CAC GAC GGG GAT GAG CAG AGC CGGCGG 672Gly Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg 210 215 220 GTG GCG CAG GTCATCATC CCC AGC ACG TAC GTC CCG GGC ACC ACC AAC 720Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn 225 230 235 240 CAC GAC ATC GCG CTG CTC CGC CTG CAC CAG CCC GTG GTC CTC ACT GAC 768His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp 245 250 255 CATGTG GTG CCC CTC TGC CTG CCC GAA CGG ACG TTC TCT GAG AGG ACG816His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg

Thr 260 265 270 CTG GCC TTC GTG CGC TTCTCATTG GTC AGC GGC TGG GGC CAG CTG CTG 864Leu Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu 275 280 285 GACCGT GGC GCC ACG GCC CTG GAG CTC ATG GTG CTC AAC GTG CCC CGG 912Asp Arg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg 290 295 300 CTGATG ACC CAG GAC TGC CTG CAG CAG TCA CGG AAG GTG GGA GAC TCC 960Leu Met Thr Gln Asp Cys Leu Gln Gln Ser Arg Lys Val Gly Asp Ser 305 310 315 320 CCA AAT ATC ACG GAG TAC ATG TTC TGT GCC GGC TAC TCG GAT GGC AGC 1008Pro Asn Ile Thr Glu Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser 325 330 335 AAGGAC TCC TGC AAG GGG GAC AGT GGA GGC CCA CAT GCC ACC CACTAC 1056Lys Asp Ser Cys Lys Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr 340 345 350 CGG GGC ACG TGG TAC CTG ACG GGC ATC GTC AGC TGG GGC CAG GGC TGC 1104Arg Gly Thr Trp Tyr-Leu-Thr-Gly-Ile Val Ser Trp Gly Gln-Gly-Cys 355 360 365 GCA-ACC-GTG-GGC-CAC TTT GGG GTG TAC ACC AGG GTC TCC CAG TAC ATC 1152Ala Thr Val Gly His Phe Gly Val Tyr Thr Arg Val-Ser-GlnTyr Ile 370 375 380 GAGTGG CTG CAA AAG CTC ATG CGC TCA GAG CCA CGC CCA GGA GTC CTC1200Glu Trp Leu Gln Lys Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu 385 390 395 400 CTG CGA GCC CCA TTT CCC TAG 1221Leu Arg AlaPro Phe Pro 405 <210> 4 <211> 406<212> PRT<213> artificail sequence<220> <223> Amino acid sequence of recombinant mutantof blood coagulation factor VII in which both of the 159th Cysteine and the 164thCysteine arereplaced with Alanine.<400> 4Ala Asn Ala Phe Leu Glu Glu Leu Arg Pro Gly SerLeu Glu Arg Glu 1 5 10 15 Cys Lys Glu Glu Gln Cys SerPhe Glu Glu Ala Arg Glu Ile Phe Lys 20 25 30 Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp GlyAsp 35 40 45Gln Cys Ala Ser Ser Pro CysGln Asn Gly Gly Ser Cys Lys Asp Gln 50 55 60Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu GlyArg Asn 65 70 75 80 Cys GluThr HisLys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly 85 9095 Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys 100 105 110 Arg Cys His GluGly TyrSer Leu Leu Ala Asp Gly Val Ser Cys Thr 115 120 125 Pro Thr Val GluTyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg 130 135 140Asn Ala Ser Lys Pro Gln Gly Arg Ile Val GlyGly Lys Val Ala Pro 145 150 155 160 Lys Gly Glu Ala Pro Trp Gln Val Leu Leu Leu Val Asn Gly Ala Gln 165 170 175 Leu-Cys-Gly-Gly-Thr Leu Ile Asn Thr Ile-Trp-Val-Val-Ser-Ala-Ala 180 185 190 His-Cys-Phe-Asp-Lys Ile Lys Asn Trp Arg-Asn-Leu-Ile-Ala-Val-Leu 195200 205Gly Glu HisAsp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg 210 215220 ValAla Gln Val Ile Ile Pro Ser Thr TyrVal Pro Gly Thr Thr Asn 225 230 235 240 His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp 245 250255 HisVal Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr 260265 270Leu Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu 275 280 285 AspArg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg 290 295 300 Leu Met Thr Gln Asp Cys Leu Gln Gln Ser Arg Lys Val Gly Asp Ser 305310 315320 ProAsn Ile Thr Glu Tyr Met Phe Cys Ala Gly Tyr 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ACC CTG ATC AAC ACC ATC TGG GTG GTC TCCGCG GCC576Leu Cys Gly Gly Thr Leu Ile
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912Asp Arg Gly Ala Thr Ala Leu Glu Leu Met Cys Leu Asn Val Pro Arg 290 295 300 CTGATG ACC
CAG GAC TGC CTG CAG CAG TCA CGGAAG GTG GGA GAC TCC 960Leu Met Thr Gln Asp
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CCA CAT GCC ACC CAC TAC 1056Lys Asp Ser Cys Lys Gly Asp Ser Gly Gly Pro His Ala Thr His
Tyr 340 345 350 CGG GGC ACG TGG TAC CTG ACG GGC ATC GTC AGC TGGGGC CAG GGC
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Gly-His Phe Gly Val Tyr Thr-Arg-Val-Ser-Gln-Tyr-Ile 370 375 380 GAG-TGG-CTG-CAA-AAG
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40 45 Gln Cys AlaSer Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln 50 5560 Leu Gln Ser Tyr Ile
Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn 65 70 75 80Cys Glu Thr His LysAsp Asp Gln Leu Ile
Cys Val Asn Glu Asn Gly 85 9095 Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser
Cys 100 105 110 Arg Cys HisGlu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr 115 120 125 Pro
Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg 130135 140Asn Ala Ser Lys Pro Gln
Gly Arg Ile Val Gly Gly Lys Val Cys Pro 145 150 155 160Lys Gly Glu Ala Pro Trp Gln Val Leu Leu
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Ala 180 185 190 His-Cys-Phe-Asp-Lys Ile Lys Asn Trp Arg-Asn-Leu-Ile-Ala-Val-Leu 195 200 205
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Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn 225 230 235240 His AspIleAla Leu Leu Arg Leu His Gln
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AAG-GAG-GAG-CAG TGC TCC TTC GAG GAG-GCC-CGG-GAG-ATC-TTC-AAG 96Cys Lys
Glu GluGln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys 20 25 30 GAC GCG GAG AGG ACGAAG
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Val Asn Glu Asn Gly 85 90 95 GGC TGT GAG CAG TAC TGC AGT GAC CAC ACG GGC ACC AAGCGC TCCTGT 336Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys 100 105 110 CGG TGC CAC GAG GGG TAC TCT CTG CTGGCA GAC GGG GTG TCC TGC ACA 384Arg Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr 115 120 125 CCCACA GTT GAA TAT CCA TGT GGA AAA ATA CCT ATT CTA GAA AAAAGA 432Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg 130 135 140 AAT GCC AGC AAACCCCAA GGC CGA ATT GTG GGG GGC AAG GTG TGC CCC 480Asn Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Cys Pro 145 150 155 160 AAA GGG GAG TGT CCA TGG CAG GTC CTG TTG TTG GTG AAT GGA GCT CAG 528Lys Gly Glu Cys Pro Trp Gln Val Leu Leu Leu Val Asn Gly Ala Gln 165 170 175 TTGTGT GGG GGG ACC CTG ATC AAC ACC ATC TGG GTG GTC TCC GCG GCC576Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala 180 185 190 CAC TGT TTC GAC AAA ATCAAGAAC TGG AGG AAC-CTG-ATC-GCG-GTG CTG 624His Cys Phe-Asp-Lys-Ile-Lys Asn Trp Arg Asn 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threonine are replaced with Asp-Arg-Lys-Thr-Leu and the 12 amino acid residues from the 311th leucine to 322th asparagine are replaced with Glu-Ala-Ser-Tyr-Pro-Gly-Lys and and cDNA sequence coding.[thereof] <400> 11GCC AAC GCG TTC CTG GAG GAG CTGCGG CCG GGC TCC CTG GAGAGG GAG 48Ala Asn Ala Phe Leu Glu Glu Leu Arg Pro Gly Ser Leu Glu Arg Glu 1 5 10 15 TGC-AAG-GAG-GAG-CAG TGC TCC TTC GAG GAG-GCC-CGG-GAG-ATC-TTC-AAG 96Cys Lys Glu Glu Gln Cys Ser Phe Glu Glu-Ala-Arg-Glu-Ile Phe Lys 20 25 30 GAC-GCG GAG AGG ACG AAG CTG TTC TGG ATTTCT TAC AGT GAT GGG GAC 144Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp 35 40 45 CAG TGT GCC TCA AGT CCA TGC CAG AAT GGG GGC TCC TGC AAG GAC CAG 192Gln CysAla Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln 50 55 60 CTC CAG TCC TAT ATC TGC TTC TGC CTC CCT GCC TTC GAG GGC CGG AAC 240Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn 65 70 75 80 TGTGAG ACG CAC AAG GAT GAC CAG CTG ATC TGT GTG AAC GAG AAC GGC 288Cys Glu Thr His Lys 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replaced with Asp-Arg-Lys-Thr-Leu and the 12 amino acid residues from the 311th leucine to 322th

asparagine are replaced with Glu-Ala-Ser-Tyr-Pro-Gly-Lys.<400> 12Ala Asn Ala Phe Leu Glu Glu Leu Arg Pro Gly Ser Leu Glu Arg Glu 1 5 10 15 Cys Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys 20 25 30 Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp35 40 45Gln Cys Ala Ser Ser Pro Cys GlnAsn Gly Gly Ser Cys Lys Asp Gln 50 55 60Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu GlyArg Asn 65 70 75 80 Cys Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly 85 9095 Gly CysGlu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys 100 105 110 Arg Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr 115 120 125 Pro Thr Val GluTyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg 130 135 140Asn Ala Ser Lys Pro Gln Gly Arg Ile Val GlyGly Lys Val Cys Pro 145 150 155 160 Lys Gly GluCys Pro Trp Gln Val Leu Leu Leu Val Asn Gly Ala Gln 165 170 175 Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala 180 185190 HisCys Phe Asp Lys Ile-Lys-Asn-Trp-Arg Asn Leu Ile Ala Val-Leu 195 200 205 Gly-Glu-His-Asp-Leu Ser Glu His Asp Gly-Asp-Glu-Gln-Ser-Arg-Arg 210 215 220 Val Ala Gln Val Ile Ile Pro Ser Thr Tyr AspArg Lys Thr LeuAsn225 230235 240His Asp Ile Ala LeuLeu Arg Leu His Gln Pro Val Val Leu Thr Asp 245 250255 HisValVal Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr 260 265 270 Leu Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu 275 280 285 Asp Arg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg 290 295 300Leu Met Thr Gln Asp Cys Glu Ala Ser Tyr Pro Gly Lys Ile Thr Glu 305 310 315 320Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser Lys Asp Ser Cys Lys 325 330 335 Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr Arg Gly Thr Trp Tyr 340 345350 LeuThr Gly Ile Val Ser Trp Gly Gln Gly Cys Ala Thr Val Gly His 355 360 365Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile Glu Trp Leu Gln Lys 370 375 380 Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu Leu Arg Ala Pro Phe 385 390 395400 Pro

Field

[The technical field to which invention belongs] The invention in this application relates to the alteration field of the blood coagulation factor VII (FVII may be called hereafter) which reinforced enzyme activity, and/or activation blood coagulation factor VII (FVIIa may be called hereafter). In detail, the invention in this application relates to a medicine effective in the treatment of the hemophilia inhibitor patient who consists of a drug constituent which contains the FVII/FVIIa alteration field with which activity was reinforced, and the concerned alteration field as an active principle, and the concerned drug constituent by replacing and suffering a loss in an amino acid sequence peculiar to FVII.

Effect

[Effect of the Invention] Thus, the alteration field of FVII obtained by the invention in this application and/or FVIIa has clearly high enzyme activity compared with FVII of a wild type. Therefore, the alteration field of the invention in this application may serve as the very effective medicine as a replacement therapy to a hemophilia inhibitor patient.
[Layout Table]
SEQUENCE-LISTING<110> The-Chemo-Sero-Therapeutic Research-Institute<120> Recombinant-mutants of-blood-coagulation factor VII<160> 12<210> 1<211> 1221<212> DNA<213> blood coagulation factor VII<400> 1GCC AAC GCG TTC CTG GAG GAG CTG CGG CCG GGC-TCC-CTG GAG AGG GAG 48Ala Asn Ala Phe Leu Glu Glu Leu Arg Pro Gly Ser Leu Glu Arg Glu 15 1015 TGC AAG GAGGAG CAG TGC TCC TTC GAG GAG GCC CGG GAG ATC TTC AAG 96Cys Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys 20 25 30 GAC GCG GAG AGG ACG AAG CTG TTC TGG ATTTCT TAC AGT GAT GGG GAC 144AspAla Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp 35 40 45 CAG TGT GCC TCA AGT CCA TGC CAG AAT GGG GGC TCC TGC AAG GAC CAG 192Gln Cys Ala Ser SerPro Cys Gln Asn Gly GlySer Cys Lys Asp Gln 50 55 60 CTC CAG TCC TAT ATC TGC TTC TGC CTC CCT GCC TTC GAG GGC CGG AAC 240Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn 65 70 75 80TGT GAG ACG CAC AAG GAT GAC CAG CTG ATC TGT GTG AAC GAG AAC GGC288Cys Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly 85 90 95 GGC TGT GAG CAG TAC TGC AGT GAC CAC ACGGGC ACC AAG CGC TCC TGT 336Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys 100 105 110 CGG TGC CAC GAG GGG TAC TCT CTG CTG GCA GAC GGG GTG TCC TGC ACA 384Arg Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr 115 120 125 CCCACA GTT GAA TAT CCA TGT GGA AAAATA CCT ATT CTAGAA AAA AGA

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GAC 768His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp 245 250 255 CATGTG GTG CCC CTC TGC CTG CCC GAA CGG ACG TTC TCT GAG AGG ACG816His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr 260 265 270 CTG GCC TTC GTG CGC TTCTCATTG GTC AGC GGC TGG GGC CAG CTG CTG 864Leu Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu 275 280 285 GACCGT GGC GCC ACG GCC CTG GAG CTC ATG GTG CTC AAC GTG CCC CGG 912Asp Arg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg 290 295 300 CTGATG ACC CAG GAC TGC CTG CAG CAG TCA CGG AAG GTG GGA GAC TCC 960Leu Met Thr Gln Asp Cys Leu Gln Gln Ser Arg Lys Val Gly Asp Ser 305 310 315 320 CCA AAT ATC ACG GAG TAC ATG TTC TGT GCC GGC TAC TCG GAT GGC AGC 1008Pro Asn Ile Thr Glu Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser 325 330 335 AAG-GAC-TCC-TGC-AAG GGG-GAC-AGT-GGA-GGC CCA CAT GCC ACC CAC TAC 1056Lys Asp Ser Cys Lys Gly Asp-Ser-Gly-Gly-Pro-His-Ala-Thr-His-Tyr 340 345 350 CGG-GGC-ACG-TGG-TAC CTG-ACG-GGC-ATC-GTCAGC TGG GGC CAG GGC 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390 395 400 CTG CGA GCC CCA TTT CCC TAG 1221Leu Arg AlaPro Phe Pro 405 <210> 4 <211> 406<212> PRT<213> artificail sequence<220> <223> Amino acid sequence of recombinant mutantof blood coagulation factor VII in which both of the 159th Cysteine and the 164thCysteine arereplaced with Alanine.<400> 4Ala Asn Ala Phe Leu Glu Glu Leu Arg Pro Gly SerLeu Glu Arg Glu 1 5 10 15 Cys Lys Glu Glu Gln Cys SerPhe Glu Glu Ala Arg Glu Ile Phe Lys 20 25 30 Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp GlyAsp 35 40 45Gln Cys Ala Ser Ser Pro CysGln Asn Gly Gly Ser Cys Lys Asp Gln 50 55 60Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu GlyArg Asn 65 70 75 80 Cys GluThr HisLys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly 85 9095 Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys 100 105 110 Arg Cys His GluGly TyrSer Leu Leu Ala Asp Gly Val Ser Cys Thr 115 120 125 Pro Thr Val GluTyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg 130 135 140Asn Ala Ser Lys Pro Gln Gly Arg Ile Val GlyGly 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Thr LeuIle Asn Thr Ile Trp Val Val Ser Ala Ala 180 185 190His Cys Phe Asp Lys Ile Lys Asn

Trp Arg Asn Leu Ile Ala Val Leu 195 200 205 Gly Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg 210 215 220 Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn 225230 235240 His Asp Ile Ala LeuLeu Arg Leu His Gln Pro Val Val Leu Thr Asp 245 250255 HisVal Val Pro Leu CysLeu Pro Glu Arg Thr PheSer Glu Arg Thr 260 265 270 Leu Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu 275 280285 Asp Arg GlyAla Thr Ala Leu Glu Leu Met Cys Leu Asn Val Pro Arg 290 295300 LeuMet Thr Gln Asp CysLeu Gln GlnSer Arg Lys Val Gly Asp Ser 305 310 315 320Pro Asn Ile Thr Glu TyrMet Phe Cys Ala Gly Tyr Ser Asp Gly Ser 325 330 335Lys Asp Ser Cys Lys Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr 340 345 350Arg Gly Thr Trp Tyr Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys 355 360 365 Ala Thr Val GlyHis Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile 370 375 380Glu Trp LeuGln Lys Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu 385 390 395400 Leu ArgAla Pro Phe Pro 405 <210> 7<211> 1221<212> 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Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly 85 90 95 GGC TGT GAG CAG TAC TGC AGT GAC CAC ACG GGC ACC AAG CGC TCC TGT 336Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys 100 1 05 110 CGG-TGC-CAC-GAG-GGG TAC-TCT-CTG-CTG-GCA GAC GGG GTG TCC TGC ACA 384Arg Cys His Glu Gly Tyr Ser-Leu-Leu-Ala-Asp-Gly-Val-Ser-Cys-Thr 115 120 125 CCC ACA GTT GAA TAT CCA TGT GGA AAA ATACCT ATT CTA GAA AAA AGA 432Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg 130 135 140 AATGCC AGC AAA CCC CAA GGC CGA ATT GTG GGG GGC AAG GTG TGC CCC480Asn Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Cys Pro 145 150 155 160 AAA GGG GAG TGT CCA TGG CAG GTC CTG TTG TTG GTG AAT GGA GCT CAG 528Lys Gly Glu Cys Pro Trp Gln Val Leu Leu Leu Val Asn Gly Ala Gln 165 170 175TTG TGT GGG GGG ACC CTG ATC AAC ACC ATC TGG GTG GTC TCC GCGGCC 576Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala 180 185 190 CAC TGT TTC GAC AAA ATCAAGAAC TGG AGG AAC CTG ATC GCG GTG CTG 624His Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu 195 200 205 GGCGAG CAC GAC CTC AGC GAG CAC GAC GGG GAT GAG CAG AGC CGG CGG 672Gly Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg 210 215 220 GTGGCG

CAG GTC ATC ATC CCC AGC ACG TAC GACAGG AAG ACT CTG AAC 720Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Asp Arg Lys Thr Leu Asn 225 230 235 240 CAC GAC ATC GCG CTG CTC CGC CTG CAC CAG CCC GTG GTC CTC ACT GAC 768His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp 245 250 255 CATGTG GTG CCC CTC TGC CTG CCC GAA CGG ACG TTC TCT GAG AGG ACG816His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr 260 265 270 CTG GCC TTC GTG CGC TTCTCATTG GTC AGC GGC TGG GGC CAG CTG CTG 864Leu Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu 275 280 285 GACCGT GGC GCC ACG GCC CTG GAG CTC ATG GTG CTC AAC GTG CCC CGG 912Asp Arg Gl y-Ala-Thr-Ala-Leu-Glu Leu Met Val Leu Asn-Val-Pro-Arg 290 295 300 CTG-ATG-ACC-CAG-GAC TGC GAA GCC TCC TAC CCT GGA AAG ATC ACG GAG 960Leu Met Thr Gln Asp Cys Glu Ala Ser Tyr Pro Gly-Lys Ile Thr Glu 305 310 315 320TAC ATG TTC TGT GCC GGC TAC TCG GAT GGC AGC AAG GAC TCC TGC AAG1008Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser Lys Asp Ser Cys Lys 325 330 335GGG GACAGTGGA GGC CCA CAT GCC ACC CAC TAC CGG GGC ACG TGG TAC 1056Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr Arg Gly Thr Trp Tyr 340 345 350 CTG ACG GGC ATC GTC AGC TGG GGC CAG GGC TGC GCA ACC GTG GGC CAC 1104Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys Ala Thr Val Gly His 355 360 365 TTTGGG GTG TAC ACC AGG GTC TCC CAG TAC ATC GAG TGG CTG CAA AAG 1152Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile Glu Trp Leu Gln Lys 370 375 380 CTCATG CGC TCA GAG CCA CGC CCA GGAGTC CTC CTG CGA GCC CCA TTT 1200Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu Leu Arg Ala Pro Phe 385 390 395 400 CCC TAG 1206Proes <210> 12 <211> 401<212> PRT<213> artificial sequence<220> <223> Amino acid sequence of recombinant mutant of blood coagulation factor VII in which the 5 amino acid residues from the 235th Valine to 239ththreonine are replaced with Asp-Arg-Lys-Thr-Leu and the 12 amino acid residues from the 311th leucine to 322th asparagine are replaced with Glu-Ala-Ser-Tyr-Pro-Gly-Lys.<400> 12Ala Asn Ala Phe Leu Glu Glu Leu Arg Pro Gly Ser Leu Glu Arg Glu 1 5 10 15 Cys Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys 20 25 30 Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp35 40 45 Gln-Cys-Ala-Ser-Ser Pro Cys Gln Asn Gly-Gly-Ser-Cys-Lys-Asp-Gln 50 55 60 Leu-Gln-Ser-Tyr-Ile Cys Phe Cys Leu Pro-Ala-Phe-Glu-Gly-Arg-Asn 65 7075 80 Cys Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly 8590 95Gly Cys Glu Gln Tyr Cys Ser Asp His Thr GlyThr Lys Arg Ser Cys 100 105 110 Arg Cys His Glu Gly Tyr Ser Leu Leu Ala Asp GlyVal Ser Cys Thr 115 120 125 Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg 130 135140 Asn Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Cys Pro 145 150 155160 Lys GlyGluCys Pro Trp Gln Val Leu Leu Leu Val Asn Gly Ala Gln 165 170 175 Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala 180185 190 His Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu 195 200 205GlyGlu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg 210 215 220 Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Asp Arg Lys Thr Leu Asn 225 230 235240 HisAsp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp 245 250255 HisValVal ProLeuCys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr 260 265 270Leu Ala PheVal Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu 275 280 285AspArg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg 290 295 300Leu Met Thr Gln Asp Cys Glu Ala Ser Tyr Pro-Gly-Lys-Ile-Thr Glu 305 310 315 320 Tyr-Met-Phe-Cys-Ala Gly Tyr Ser Asp Gly-Ser-Lys-Asp-Ser-Cys-Lys 325 330 335 Gly-Asp-Ser Gly Gly Pro His Ala Thr HisTyrArg Gly Thr Trp Tyr 340 345350 LeuThr GlyIle Val Ser Trp Gly Gln Gly Cys Ala Thr Val Gly His 355 360365Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile Glu TrpLeu Gln Lys 370 375 380Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu Leu Arg Ala Pro Phe 385 390 395 400Pro

TECHNICAL PROBLEM

[A proior art and the technical probrem which should be solved] FVII is the blood coagulation factor of a vitamin K dependency, and it is known widely that it is the initiation factor of external cause system blood coagulation. It has Gla field which becomes the amino acid sequence from an amino terminus to 35 residues from ten gamma carboxy glutamic acid (Gla may be called hereafter) like other vitamin K dependency coagulation factors (vol. Proc.Natl.Acad.Sci.USA, 83, p.2412- 2416, 1986). FVII is set to in vitro. The activation blood coagulation Xth factor By (FXa may be called hereafter), activation blood coagulation factor IX (FIXa may be called hereafter), or the thrombin (FIIa may be called hereafter) Being changed, active FVII, i.e., FVIIa, which consists of an H chain by which 152Arg-153Ile was understood an added water part, and the bridge was constructed over it by the S-S bond of a piece, and an L chain, is known (J. Biol.Chem., vol. 251, p.4797- 4802, 1976).

[0003] If the enzyme activity of the FVIIa [itself] is very weak and it combines with the tissue factor

(TF) which is a coenzyme, it will go up dramatically (Komiyama et al., Biochemistry, 29 (40), and pp.9418-25 (1990)). Although the bonding site between biparite children is also made clear on amino-acid-residue level to the primary structure of FVIIa and TF, the crystal structure of the complex, and the pan, the detail (spacial-configuration change accompanied by TF combination) of the catalytic activity multiplication mechanism is still unknown (Banner et al., et al., and Nature 380(6569):pp.41-6 (1996)).

[0004] As a replacement therapy to hemophilia A and a hemophilia B patient, medication of blood coagulation factor VIII (FVIII may be called hereafter) and a blood coagulation factor IX (FIX may be called hereafter) tablet is performed. However, in connection with the concerned cure, the occurrence of the neutralizing antibody (called an inhibitor) to FVIII and FIX is regarded as questionable.

[0005] As a symptomatic treatment of the hemophiliac who produced such an inhibitor, there are medication of the complex tablet which consists of medication of superfluous medication of (1) FVIII factor and a (2) swine FVIII factor, (3) FII, and FVII, FIX and FX, medication of (4) FVIIa tablet, etc. however, such technique -- respectively -- (1) -- more -- high -- about the shock according to an antigenicity about the symptom aggravation by lead of a potency inhibitor, and (2), and (3), the cost has [4 / (4) / a thrombus induction of DIC, and] problems, such as being high, by that a curative effect is inadequate, or extensive and a frequent administration When the balance of an effect and danger is taken into consideration in these, the most efficient thing is medication of FVIIa tablet of (4). However, in order to demonstrate the hemostasis effect for the weakness of the activity, FVIIa tablet needs extensive medication and a frequent administration, as mentioned above, and is raising the treatment cost greatly. Moreover, if compared with the conventional replacement therapy to which the curative effect is also performed to the hemophiliac, it cannot be said that it is enough.

[0006] Although producing the alteration field of FVII which raised enzyme activity as a means for solving this problem is mentioned, it is known that this is generally difficult (proteinic structure admission, Yukiteru Katsube editorial supervisions, educational company issue, 1992). It is considered by the ground [blood coagulation factor] of the following especially for the activity reinforcement by alteration to be difficult.

[0007] It is classified into two of the activity falls depended qualitatively unusually with the activity fall accompanied by a quantitative deficit although hemophilias are the abnormalities of a blood coagulation factor. Among these, masses [the example from which it becomes clear that the abnormalities in a molecule exist over the structure whole region of FIX, only one amino acid was only replaced by inside, and activity becomes 1% or less] as a result of knowing that many of qualitative abnormalities are mutations (point) and performing analysis of the patient of the hemophilia B which is the abnormalities of FIX. Therefore, even if it changes recklessly about a blood coagulation factor, it is clear to cause an activity fall.

[0008] According to the information (Dickinson et al., Proc.Natl.Acad.Sci.USA, 93 (25), and pp.14379-84 (1996)) acquired by Alanine Scannning, 112 Alanine substitution-product ******s of FVII and the thing which enzyme activity went up in it are only [one], and, moreover, the regularity is not found out.

[0009] As other attempts, Hopfner et al. produced FIX fragmentation alteration field which raised synthetic substrate activity using the technique of suffering for it a loss and replacing the structural unit which consists of a number amino acid residue of the domain of the part which constitutes FIX (EMBO J, 16 (22), and pp.6626-35 (1997)). However, since this made the intact partial fragmentation of not FIX but FIX discover by Escherichia coli and is looking at synthetic substrate activity, not to mention it can reinforce blood coagulation activity, it does not have even blood coagulation activity. Furthermore, this is not suggested at all to FVII which is the quality of a different thing from which structure and a specificity are completely different about FIX, and there is no report in any way until now also about the alteration field which reinforced the enzyme activity of FVII.

[0010] Thus, production of the alteration field which has strong enzyme activity was considered to be difficult especially in the blood coagulation factor. In FIX, although the attempt which raises synthetic substrate activity about the partial fragmentation was made, about the alteration field of the blood coagulation factor which has enzyme activity high as an intact molecule, there is no example of a report until now.

[0011] Therefore, the technical probrem which should solve this invention is producing and offering FVII and/or FVIIa which have strong activity effective in a hemophilia inhibitor patient's treatment in the status considered that an alteration of a blood coagulation factor is difficult generally.

MEANS

[Means for Solving the Problem] In the above statuses, this invention persons came to complete the invention in this application, as a result of repeating a research zealously that FVII which has enzyme activity high in itself should be produced and performing various studies. It succeeds in the invention in this application producing FVII and/or FVIIa alteration field with which activity was reinforced by comparing FVII and various serine proteases with amino-acid-sequence structure, clarifying an amino-acid-sequence site peculiar to FVII, and suffering for it a loss and replacing the characteristic site.
[0013]
[Elements of the Invention] a group similar to a trypsin group -- the basic structure of a serine protease consists of about 250 residues, and is about divided into two domains, the first half and the second half, on an amino acid sequence (drawing 1) Six beta strands are in each domain, respectively, and it is formed with the structure of having beta strand of a total of 12 as a protease (drawing 2). So to speak, these 12 beta strands have the skeletal structure of a serine protease, and the loop which connects between each strand, or the helix field is considered to bear protease activity, such as the substrate specificity and reactivity with a cofactor. As an example of a serine protease, there are digestive enzymes, such as thrombus lysis enzymes, such as various blood coagulation factors, such as FII, FVII, FVIII, FIX, and FX, and a plasmin, or a trypsin, a chymotrypsin, and an elastase. Then, amino-acid-sequence structure of the various serine proteases including FVII was compared, and the field characteristic of FVII was pinpointed (drawing 3). And it considered as the target of an alteration of these sites, and FVII alteration field which has high enzyme activity was produced by suffering for it a loss and replacing the amino acid sequence of FVII for the structure of other serine proteases by reference. These alteration fields are explained in detail.
[0014] (a) The alteration field with which the concerned 159Cys-164Cys was cut by replacing alteration field (a-**) 159Cys from which 159Cys-164Cys combination was cut, and 164Cyses by amino acid residues other than Cys (VII-5). As an example of this alteration field, what replaced Cys by the alanine (Ala), respectively was indicated for the array table array numbers 3 or 4. Here, as an example of amino acid residues other than Cys used for a substitute, although Ala was chosen, unless a serious failure of making enzyme activity deactivate besides cutting Cys-Cys combination etc. is done by the substitute, arbitrary amino acid is selectable.
[0015] (a-**) The alteration field with which 159Cys-164Cys was cut and the disulfide bond (159Cys-299Cys) was formed between 159Cyses and 299Cyses by amino acid residues other than Cys replacing 164Cys, and replacing the 299th ******** (299Val) by Cys (VII-6). What was replaced as an example of this alteration field, using Ala as amino acid residues other than Cys was indicated for the array table array numbers 5 or 6. Unless a serious failure of making enzyme activity deactivate besides cutting 159Cys-164Cys combination by the substitute etc. is done about an amino acid residue except Cys used for a substitute here as above-mentioned, other amino acid other than Ala is selectable.
[0016] (b) The alteration field with which the amino acid sequence which constitutes the loop structure (99-loop may be called hereafter) which consists of an amino acid sequence of the 240th asparagine (240Asn) from the 233rd ********** (233Thr) in FVII, or its part was replaced, added or deleted.
This field contains the amino acid sequence which intervenes between the beta strand 5 which exists common to a serine protease as shown in drawing 3 , and the beta strand 6. It is desirable to replace this field by the amino acid sequence which corresponds on the structure of other trypsin group serine proteases. A Homo-sapiens trypsin is mentioned as a suitable example of a trypsin group serine protease. Furthermore, the alteration field (VII-30) with which the amino acid sequence from the 235th valine in 99-loop of FVII (235Val) to the 239th ********** (239Thr) was replaced by Asp-Arg-Lys-Thr-Leu in the loop structure of a trypsin as a concrete example is mentioned. This alteration field was indicated for the array table array numbers 7 or 8.
[0017] (c) The alteration field with which the amino acid sequence which constitutes the mediation amino acid sequence of the 329th cysteine (329Cys) from the 304th arginine (304Arg) in FVII, or its part was replaced, added or deleted.
the amino acid sequence which intervenes between the beta strand 8 which exists common to a serine protease, and the beta strand 9 as especially this field is shown in drawing 3 -- setting -- the serine protease of others [FVII] -- comparing -- a number amino acid residue -- since it has the characteristic feature of being long, what may serve as the suitable target in FVII alteration is conjectured It is desirable to replace this field by the amino acid sequence which corresponds on the structure of other trypsin group serine proteases. A Homo-sapiens trypsin is mentioned as a suitable example of a trypsin group serine protease. Moreover, the substitute in FVII and the desirable field which is added and can be deleted are the amino acid sequence which constitutes the loop structure (170-loop may be called) which consists of an amino acid sequence of the 329th cysteine (329Cys) from the 310th cysteine

(310Cys), or its part. Furthermore, the alteration field (VII-31) with which the amino acid sequence from the 311st leucine in 170-loop of FVII (311Leu) to the 322nd asparagine (322Asn) was replaced by Glu-Ala-Ser-Tyr-Pro-Gly-Lys in the loop structure of a Homo -sapiens trypsin as a concrete example is mentioned. This alteration field was indicated for the array table array numbers 9 or 10.

[0018] Furthermore, it is also possible to combine an alteration of (c) suitably from the above (a). the example ****** -- for example, (b) and the combination of (c) -- that is The amino acid sequence from the 235th valine in 99-loop of FVII (235Val) to the 239th ********** (239Thr) It is replaced by Asp-Arg-Lys-Thr-Leu in the loop structure of a Homo -sapiens trypsin. And the amino acid sequence from the 311st leucine in 170-loop (311Leu) to the 322nd asparagine (322Asn) The alteration field (VII-39) replaced by Glu-Ala-Ser-Tyr-Pro-Gly-Lys in the loop structure of a trypsin is mentioned. This alteration field was indicated for the array table array numbers 11 or 12.

[0019] The alteration field mentioned above can be acquired using the recombining [a gene] method. As a manifestation recipient, eukaryotic cells, such as an animal cell, are desirable. The alteration field of this invention includes cDNA which carries out the code of the amino acid sequence of each above-mentioned alteration field in a suitable manifestation vector, transfects a host cell, and is acquired by refining after carrying out the cloning of the cell which has discovered the target gene and cultivating the obtained stable manifestation stock.

[0020] FVII alteration field of the invention in this application can perform various chemical treatments etc., and can use them as activated type FVII (FVIIa) alteration field.

[0021] The FVII/FVIIa alteration field of the invention in this application can be prescribed to a pharmaceutical preparation because of the treatment, a diagnosis, or other intended use. To the preparation for intravenous administration, it melts into the aqueous solution which has buffered pH which may suit a physiological conditions in a constituent, including the matter which may usually suit physiologically, for example, a sodium chloride, a glycine, etc. Moreover, from the viewpoint of reservation of long term stability, as a final pharmaceutical form, taking the gestalt of a freeze-drying tablet is also taken into consideration, and it gets. In addition, the guideline of the constituent prescribed for the patient into the vena is established under the governmental rule, for example, "biological-preparation criteria." The treatment of the hemophilia inhibitor patient who produced the inhibitor to the concerned blood coagulation factor by the replacement therapy of FVIII or FIX as concrete intended use of the drug constituent which consists of the FVII/FVIIa alteration field of the invention in this application is mentioned.

EXAMPLE

[Example] Although the invention in this application is illustrated according to an example, these examples do not limit the invention in this application. With reference to an accompanying drawying, it illustrates in the example [****] about the invention in this application. An example makes the alteration field discover in the culture supernatant of an animal cell (CHO-K1). The reagent in connection with [as long as there is no notice especially the following] transgenics etc. is TAKARA SHUZO, Toyobo, and par ********** applied New England Biolabs. The product of a shrine was used.

[0023] The cloning>> Homo -sapiens liver cDNA library (TAKARA SHUZO) of <<example 1.FVIIcDNA is purchased. Reference () etc. [Molecular Basis] of Thrombosia and cDNA array well-known at Hemostasis (-- FVII synthetic DNA sense primer (VII-PWN;GGGGTCGACATGGTCTCCCAGGCCCTCAGGCTCCTCTGCCTTCTG) which added SalI site to the array table array number 1 on the basis of written) -- and PCR is performed using the anti sense primer (VII-PWC;CCCGGATCCCTAGGGAAATGGGGCTCGCAGGAGGACTCCTGGGCG) which added BamHI site. The cloning was carried out to commercial cloning vector pCRII (Invitrogen). In this case, DNA sequence was performed by the conventional method and it checked having a well-known array (Hagen FS et al and PNAS 1986; 83; 2412-6) by reference etc.

[0024] Manufacture>> manifestation vector pCAGn (patent official report of No. 2824434) of a <<example 2.FVII manifestation vector was digested by SalI and BamHI, the ligation of what cut the DNA fragment prepared in the above-mentioned example 1 containing the array which carried out the code of the FVII there by SalI and BamHI was carried out, the transformation was carried out to Escherichia coli JM105, it cultivated on LB agar medium of ampicillin inclusion, and transformation Escherichia coli was chosen. It cultivated by the culture medium of marketing of the colony which appeared overnight, extraction refining of the target manifestation plasmid was carried out, and "pVII-

W" was prepared. DNA sequence of this manifestation vector was performed and it checked having the target gene sequence.

[0025] Each FVII alteration field which has the amino acid sequence shown in manufacture>> view 4 of a <<example 3. alteration field manifestation vector was created by the following technique. In addition, drawing 4 is what showed only the amino acid sequence by the side of the end of C from the 153rd isoleucine of FVII, and about the amino acid by the side of the end of N, an alteration is not performed but is all the same than the 152nd arginine as that of a wild type. PCR is performed, using FVII gene as mold using the synthetic DNA primer shown in drawing 5 , and each amplification fragment is obtained. The ligation of each amplification fragment and the thing which cut manifestation vector pCAGn by SalI and BamHI was carried out, the transformation was carried out to Escherichia coli JM105, it cultivated on LB agar medium of ampicillin inclusion, and transformation Escherichia coli was chosen. It cultivated by the culture medium of marketing of the colony which appeared overnight, extraction refining of the target manifestation plasmid was carried out, and "pVII-5", "pVII-30", and "pVII-31" were prepared (drawing 6). Moreover, about "pVII-6", the gene obtained in drawing 5 using primer ** and ** of a publication is used as mold, and it was obtained by performing PCR further using primer ** and **. Moreover, about "pVII-39", the gene obtained using primer ** and (10) is used as mold, and it was obtained by performing PCR further using a primer (11) and (12). Furthermore DNA sequence was performed and it checked that these plasmids had the target array.

[0026] The commercial ******** cutin reagent performed the transduction to CHO cell, the manifestation to the culture supernatant of <<example 4. each alteration field and the refining>> above-mentioned manifestation vector were chosen by G418 (1mg/(ml)), and the cloning of the cell which has discovered the target gene was carried out with the extra dilution method. ELISA kit (****** chromium FVII;Diagnostica Strago) to commercial FVII performed authentication of a manifestation of FVII alteration field. The obtained stable manifestation stock was cultivated by the serum free medium (ASF104, Ajinomoto, penicillin, streptomycin, 20microg [/ml] vitamin K, 1mM butyric acid), and was refined in the anti-Homo-sapiens FVII monoclonal antibody column (patent official report of No. 2824430). An equilibration, washing, and elution were performed using the equilibration and the washing buffer (50mM Tris, pH 7.2, 0.1M NaCl, 50mM Benzamidine-HCl, and 2mM calcium2+), and the elution buffer (50mM Tris, pH 7.2, 0.1MNaCl, 50mM Benzamidine-HCl, and 10mM EDTA). Using the antibody [as opposed to SDS-PAGE or commercial FVII for the purified alteration field], the Western blot was performed and it checked that it was FVII alteration field.

[0027] The freezing activity of measurement>> each alteration field of the freezing activity of <<example 5. each alteration field was measured by the solidifying method using FVII lack plasma according to the conventional method. each refined alteration field is set to 50 to 5 ng/ml -- as -- Tris-BSA -- diluting -- FVII lack ****, equivalent ****, and 37 degrees C -- 3 minutes -- warming -- equivalent addition of the formation TF (thromboplastin;Dade) of a re-lipid was carried out the back, and the freezing reaction was made to start The coagulation time was measured and it asked for freezing activity from the standard curve and the dilution ratio. The result which converted freezing activity into per [protein concentration (it measures by the Bradford method)], and asked for the specific activity is described in Table 1. Consequently, FVII alteration field of this invention became clear [having freezing activity high two to 6 times] as compared with plasma origin FVII and wild-type recombination FVII.

[0028]

[Table 1]

サンプル	改変内容	凝固活性 U/ml	蛋白濃度 μg/ml	比活性 U/ml	相対比 %
血漿由来	天然品	2,000	1,000	2,000	100
VII-W	組換え野生型	3,400	1,700	2,000	100
VII-5	159Cys-164Cys の切断	4,954	1,032	4,800	240
VII-6	159Cys-299Cys の形成	6,636	1,293	5,132	257
VII-30	loop99 を Trypsin 型へ	3,361	685	4,907	245
VII-31	loop170 を Trypsin 型へ	3,589	877	4,093	205
VII-39	loop99+170 を Trypsin 型へ	8,954	773	11,584	579

[0029] Each alteration field each alteration field of which the <<example 6. activation was done did whose manufacture>> refining is dialyzed to 50mM Tris, pH 7.45, and 0.1M NaCl. FXa is added 1/100 (mole ratio). 50mM Tris, pH 7.45, 0.1M NaCl, 0.1% PEG 8000, 100microg / mL phospholipid (Platerin (registered trademark) Organotecnica), and 10mM calcium2+, Under the 37-degree C

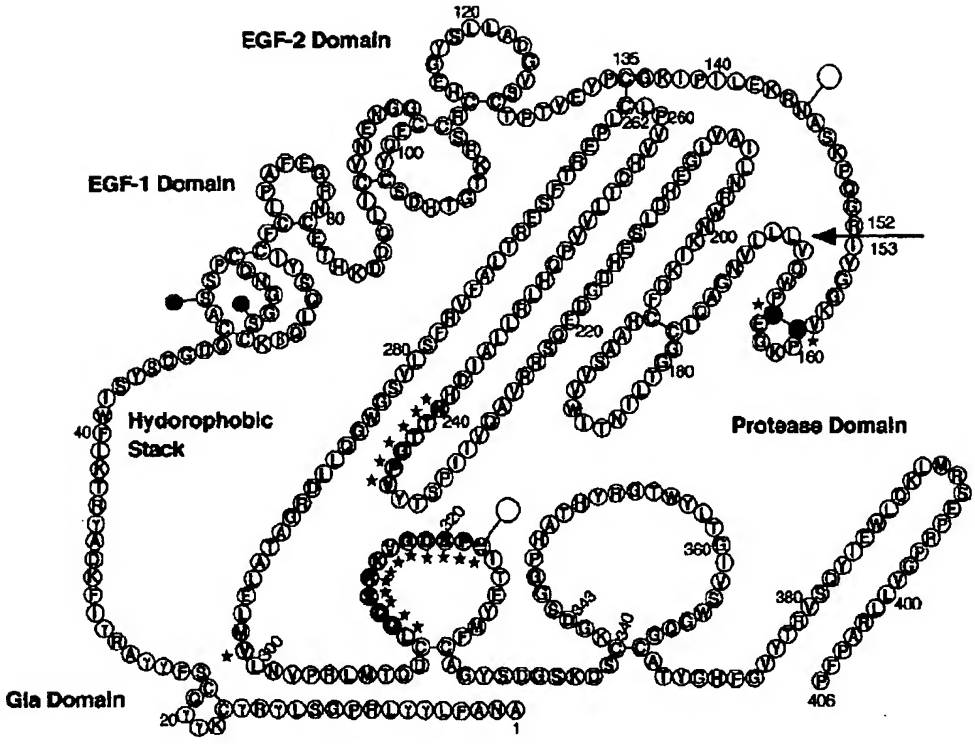
condition, in 1 - 60 minutes, the incubation was carried out and it activated. After the activation, 50mM Benzamidine-HCl was added, the reaction was stopped, and it refined in the anti-Homo-sapiens FVII monoclonal antibody column (the same technique as an example 4). Each activation alteration field [finishing / refining] was dialyzed to TBS pH 8.0 (0.1% PEG 8000 inclusion), and carried out the cryopreservation to -80 degrees C. The grade of an activation was checked by SDS-PAGE.
[0030] Alteration field VIIa-31 activated according to the hydration activity-measurement>> example 6 over the synthetic substrate of each alteration field of which the <<example 7. activation was done until it is set to 0.1microM It dilutes with 50mMTris-HCl, 100mM NaCl, 10mM calcium2+, 0.1% PEG 8000, and pH 8.0. In addition, set the last capacity to 200microl, it was made to react at 30 degrees C so that various synthetic substrates may be set to last concentration 1.0mM there, and the amount of hydrations of the substrate per for 1 minute was seen. A temperature control is possible. Disengagement of pNA was measured as a degree of coloring by 405nm by microplate reader Spectra max plus (Molecular device). This result is shown in Table 2. VIIa-31 which are one of the alteration fields of this invention also received the synthetic substrate of what **, and showed hydration activity higher than a wild type (VIIa-W), and the domain was two to 23 times.
[0031]
[Table 2]

基質名	構造	水解活性／mOD_{405nm}/min		比
		VIIa-W	VIIa-31	31/W
Chromozym tPA	D-Phe-Gly-Arg	37.6	117.6	3
S-2288	H-D-Ile-Pro-Arg	25.8	304.2	12
S-2366	pyro-Glu-Pro-Arg	11.5	267.8	23
S-2238	H-D-Phe-Pip-Arg	11.3	86.6	8
Chromozym X	D-Nile-Gly-Arg	13.0	48.6	4
S-2302	H-D-Pro-Phe-Arg	7.4	38.3	5
S-2765	Z-D-Arg-Gly-Arg	13.3	20.6	2
Chromozym TRY	CBz-Val-Gly-Arg	5.6	28.2	5
S-2444	pyro-Glu-Gly-Arg	1.5	18.9	13
S-2222	Bz-Ile-Glu-Gly-Arg	5.6	16.4	3
S-2403	pyro-Glu-Phe-Lys	0.3	5.9	19

[0032]

DESCRIPTION OF DRAWINGS

[Brief Description of the Drawings]
[Drawing 1] Drawing showing the primary structure and the alteration site (asterisk) of FVII.
[Drawing 2] Drawing showing the basic structure of the serine protease on the basis of the protease domain amino acid sequence of FVII.
[Drawing 3] Drawing showing the 3D multi-alignment between the various trypsins group serine proteases of X-ray spacial-configuration known.
[Drawing 4] Drawing showing a part of amino acid sequence of wild-type FVII (FVII-Wild) and various FVII alteration fields. This view is what showed only the amino acid sequence by the side of the end of C from the 153rd isoleucine of FVII, and about the amino acid sequence by the side of the end of N, neither performs an alteration, but it is the same than the 152nd arginine as that of a wild type.
[Drawing 5] Drawing showing the primer array for FVII alteration field production.
[Drawing 6] Drawing showing the construction technique of FVII alteration field manifestation vector.



Drawing 1

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 I V G G K V C P K G E C P W Q V L L L V N G A Q L C G G T L I N T I W V V S A A
153                          βストランド1   βストランド2       βストランド3 192

 H C F D K I K N W R N L I A V L G E H D L S E H D G D E Q S R R V A Q V I I P S
193              βストランド4                    βストランド5              232

 T Y V P G T T N H D I A L L R L H Q P V V L T D H V V P L C L P E R T F S E R T
233              βストランド6                                              272

 L A F V R F S L V S G W G Q L L D R G A T A L E L M V L N V P R L M T Q D C L Q
273      βストランド7                    βストランド8                      312

 Q S R K V G D S P N I T E Y M F C A G Y S D G S K D S C K G D S G G P H A T H Y
313                  βストランド9                        βストランド10 352

 R G T W Y L T G I V S W G Q G C A T V G H F G V Y T R V S Q Y I E W L Q K L M R
353   βストランド11                  βストランド12                    392

 S E P R P G V L L R A P F P
393
```

Drawing 2

sequence 1: ヒト血液凝固第VII因子 (pdb ID 1DAN)
sequence 2: ヒトトリプシン (pdb ID 1TRN)
sequence 3: ブタ血液凝固第IX因子 (pdb ID 1PFX)
sequence 4: ウシトリプシン (pdb ID 1TLD)
sequence 5: ヒト血液凝固第X因子 (pdb ID 1HCG)
sequence 6: ヒトプロテインC (pdb ID 1AUT)
sequence 7: ブタカリクレインA (pdb ID NPKA)
sequence 8: ウシキモトリプシン (pdb ID 5CHA)
sequence 9: ブタエラスターゼ (pdb ID 3EST)
sequence10: ヒトαトロンビン (pdb ID 1PPB)
sequence11: ヒト多形核白血球プロテアーゼ3 (pdb ID 1FUJ)
sequence12: ラットトニン (pdb ID 1TON)
sequence13: ヒト好中球エラスターゼ (pdb ID 1HNE)
sequence14: ヒトウロキナーゼ型プラスミノーゲンアクチベータ (pdb ID 1LMW)
sequence15: ヒトカテプシンG (pdb ID 1CGH)
sequence16: ラット肥満細胞プロテアーゼ (pdb ID 3RP2)
sequence17: ヒト組織型プラスミノーゲンアクチベータ (pdb ID 1RTF)

(図中@位置はすべてのプロテアーゼでのCα位置が1Å以内で保存されている構造保存部位を示す)

	βストランド5-βストランド6近傍のアライメント	βストランド8-βストランド9近傍のアライメント
	@@@@@@@@@@:@@@::::::::@@@@@@@@@@	@@@@@@@@@@@@:::::::::::::::::@@@@@@@
sequence 1:	SRRVAQVIIPSTYVP----G-TTNHDIALLRLHQ	ALELMVLNVPRLMTQDCLQQSRKVGDSPNITEYMFCAG
sequence 2:	FINAAKIIRHPQYDR----K-TLNNDIMLIKLSS	PDELQCLDAPVLSQAKCEA-S-Y---PGKITSNMFCVG
sequence 3:	RRNVIRAIPHHSYNAT---VNKYSHDIALLELDE	ATILQYLKVPLVDRATCL--R-ST--KFTIYSNMFCAG
sequence 4:	FISASKSIVHPSYNS----N-TLNNDIMLIKLKS	PDVLKCLKAPILSDSSCKS-A-Y---PGQITSNMFCAG
sequence 5:	VHEVEVVIKHNRFTK----E-TYDFDIAVLRLKT	STRLKMLEVPYVDRNSCKL-S-S---SFIITQNMFCAG
sequence 6:	DLDIKEVFVHPNYSK----S-TTDNDIALLHLAQ	TFVLNFIKIPVVPHNECSE-V-M---SNMVSENMLCAG
sequence 7:	FFGVTADFPHPGFNLSA-DGKDYSHDLMLLRLQS	PDEIQCVQLTLLQNTFCA--D-AH--PDKVTESMLCAG
sequence 8:	KLKIAKVFKNSKYNS----L-TINNDITLLKLST	PDRLQQASLPLLSNTNCKK-Y-W---GTKIKDAMICAG
sequence 9:	YVGVQKIVVHPYWNT--D-DVAAGYDIALLRLAQ	AQTLQQAYLPTVDYAICSS-SSYW--GSTVKNSMVCAG
sequence10:	ISMLEKIYIHPRYNW----RENLDRDIALMKLKK	PSVLQVVNLPIVERPVCKD-S-T---RIRITDNMFCAG
sequence11:	HFSVAQVFLN-KYDA----E-NKLNDILLIQLSS	AQVLQELNVTVVT--FFC----------R-PHNICTF
sequence12:	RRLVRQSFRHPDYIP--LPVHDHSNDLMLLHLSE	SHDLQCVNIHLLSNEKCI--E-TY--KDNVTDVMLCAG
sequence13:	VFAVQRIFED-GYDP----V-NLLNDIVILQLNG	ASVLQELNVTVVT--SLC----------R-RSNVCTL
sequence14:	KFEVENLILHKDYSA--D-TLAHHNDIALLKIRS	PEQLKMTVVKLISHRECQQPH-YY--GSEVTTKMLCAA
sequence15:	HITARRAIRHPQYNQ----R-TIQNDIMLLQLSR	TDTLREVQLRVQRDRQCLR-I-F---GSYDPRRQICVG
sequence16:	KIKVEKQIIHESYNS----V-PNLIIDIMLLKLEK	SYTLREVELRIMDEKACVD-Y-R---Y-YEYKFQVCVG
sequence17:	KFEVEKYIVHKEFDD----D-TYDNDIALLQLKS	SERLKEAHVRLYPSSRCTSQH-LL--NRTVTDNMLCAG

Drawing 3

VII-Wild

IVGGKVCPKGECPWQVLLLVNGAQLCGGTLINTIWVVSAAHCFDKIKNWRNLIAVLGEHD
LSEHDGDEQSRRVAQVIIPSTYVPGTTNHDIALLRLHQPVVLTDHVVPLCLPERTFSERT
LAFVRFSLVSGWGQLLDRGATALELMVLNVPRLMTQDCLQQSRKVGDSPNITEYMFCAGY
SDGSKDSCKGDSGGPHATHYRGTWYLTGIVSWGQGCATVGHFGVYTRVSQYIEWLQKLMR
SEPRPGVLLRAPFP

VII-5

IVGGKVAPKGEAPWQVLLLVNGAQLCGGTLINTIWVVSAAHCFDKIKNWRNLIAVLGEHD
LSEHDGDEQSRRVAQVIIPSTYVPGTTNHDIALLRLHQPVVLTDHVVPLCLPERTFSERT
LAFVRFSLVSGWGQLLDRGATALELMVLNVPRLMTQDCLQQSRKVGDSPNITEYMFCAGY
SDGSKDSCKGDSGGPHATHYRGTWYLTGIVSWGQGCATVGHFGVYTRVSQYIEWLQKLMR
SEPRPGVLLRAPFP

VII-6

IVGGKVCPKGEAPWQVLLLVNGAQLCGGTLINTIWVVSAAHCFDKIKNWRNLIAVLGEHD
LSEHDGDEQSRRVAQVIIPSTYVPGTTNHDIALLRLHQPVVLTDHVVPLCLPERTFSERT
LAFVRFSLVSGWGQLLDRGATALELMCLNVPRLMTQDCLQQSRKVGDSPNITEYMFCAGY
SDGSKDSCKGDSGGPHATHYRGTWYLTGIVSWGQGCATVGHFGVYTRVSQYIEWLQKLMR
SEPRPGVLLRAPFP

VII-30

IVGGKVCPKGECPWQVLLLVNGAQLCGGTLINTIWVVSAAHCFDKIKNWRNLIAVLGEHD
LSEHDGDEQSRRVAQVIIPSTYDRKTLNHDIALLRLHQPVVLTDHVVPLCLPERTFSERT
LAFVRFSLVSGWGQLLDRGATALELMVLNVPRLMTQDCLQQSRKVGDSPNITEYMFCAGY
SDGSKDSCKGDSGGPHATHYRGTWYLTGIVSWGQGCATVGHFGVYTRVSQYIEWLQKLMR
SEPRPGVLLRAPFP

VII-31

IVGGKVCPKGECPWQVLLLVNGAQLCGGTLINTIWVVSAAHCFDKIKNWRNLIAVLGEHD
LSEHDGDEQSRRVAQVIIPSTYVPGTTNHDIALLRLHQPVVLTDHVVPLCLPERTFSERT
LAFVRFSLVSGWGQLLDRGATALELMVLNVPRLMTQDCEASYP-----GKITEYMFCAGY
SDGSKDSCKGDSGGPHATHYRGTWYLTGIVSWGQGCATVGHFGVYTRVSQYIEWLQKLMR
SEPRPGVLLRAPFP

VII-39

IVGGKVCPKGECPWQVLLLVNGAQLCGGTLINTIWVVSAAHCFDKIKNWRNLIAVLGEHD
LSEHDGDEQSRRVAQVIIPSTYDRKTLNHDIALLRLHQPVVLTDHVVPLCLPERTFSERT
LAFVRFSLVSGWGQLLDRGATALELMVLNVPRLMTQDCEASYP-----GKITEYMFCAGY
SDGSKDSCKGDSGGPHATHYRGTWYLTGIVSWGQGCATVGHFGVYTRVSQYIEWLQKLMR
SEPRPGVLLRAPFP

(下線部は改変部位を表す)

Drawing 4

①VII-PWN Sense；5′-GGGGTCGACATGGTCTCCCAGGCCCTCAGGCTCCTCTGCCTTCTG-3′
Factor VII Wild type のシグナル配列からのプライマーデザイン
5′-GGGGTCGACATGGTCTCCCAGGCCCTCAGGCTCCTCTGCCTTCTG-3′
SalI M V S Q A L R L L C L L

②VII-PWC AntiS；5′-CCCGGATCCCTAGGGAAATGGGGCTCGCAGGAGGACTCCTGGGCG-3′
Factor VII Wild type のカルボキシ末端までのプライマーデザイン
5′-CCCGGATCCCTAGGGAAATGGGGCTCGCAGGAGGACTCCTGGGCG-3′
BamHI

③VII-P5-1 Sense；5′-ATTGTGGGGGGCAAGGTGGCCCCCAAAGGGGAGGCCCCATGGCAGGTC-3′
④VII-P5-2 AntiS；5′-GACCTGCCATGGGGCCTCCCCTTTGGGGGCCACCTTGCCCCCCACAAT-3′
VII-5のプライマーデザイン（C159A, C164A）
5′-ATTGTGGGGGGCAAGGTGGCCCCCAAAGGGGAGGCCCCATGGCAGGTC-3′
3′-TAACACCCCCCGTTCCACCGGGGGTTTCCCCTCCGGGGTACCGTCCAG-5′
I V G G K V A P K G E A P W Q V

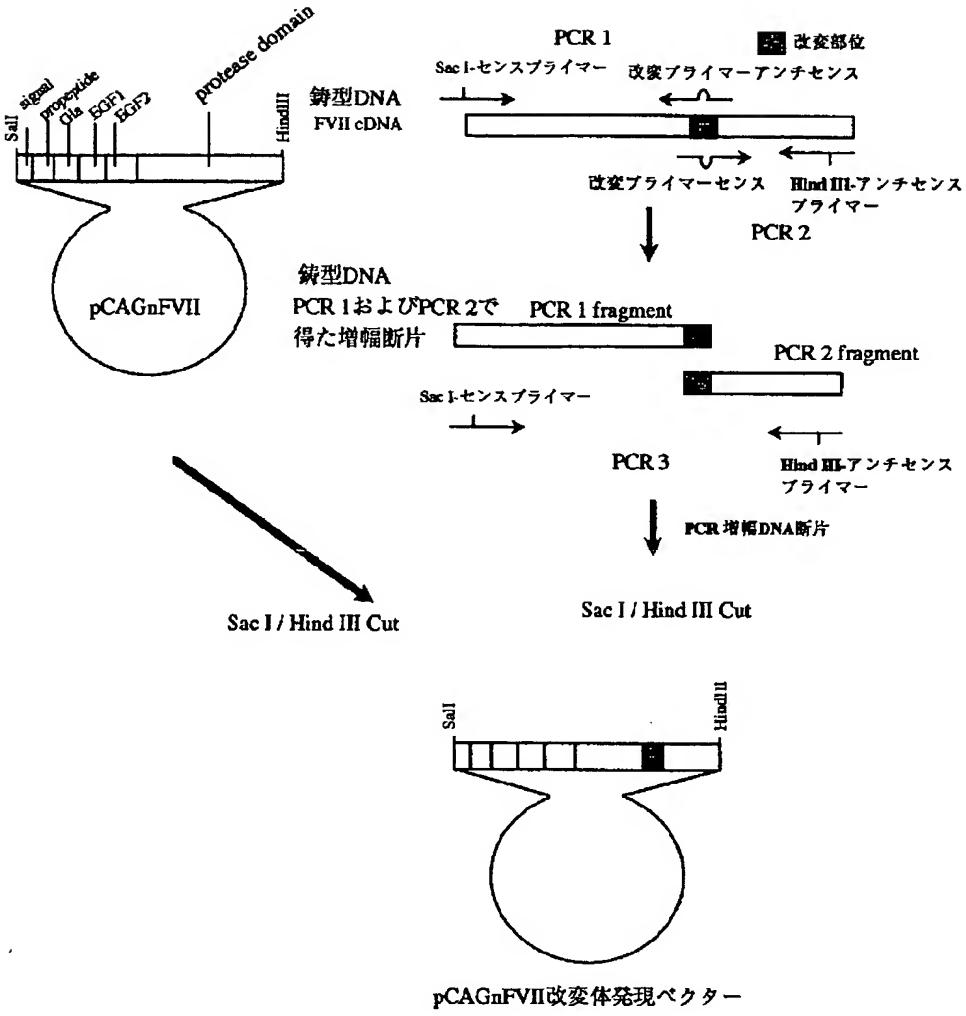
⑤VII-P6-1 Sense；5′-TGCCCCAAAGGGGAGGCCCCATGGCAGGTC-3′
⑥VII-P6-2 AntiS；5′-GACCTGCCATGGGGCCTCCCCTTTGGGGCA-3′
VII-6のプライマーデザイン① （C164A）
5′-TGCCCCAAAGGGGAGGCCCCATGGCAGGTC-3′
3′-ACGGGGTTTCCCCTCCGGGGTACCGTCCAG-5′
C P K G E A P W Q V

⑦VII-P6-3 Sense；5′-CTGGAGCTCATGTGCCTCAACGTGCCCCGG-3′
⑧VII-P6-4 AntiS；5′-CCGGGGCACGTTGAGGCACATGAGCTCCAG-3′
VII-6のプライマーデザイン② （V299C）
5′-CTGGAGCTCATGTGCCTCAACGTGCCCCGG-3′
3′-GACCTCGAGTACACGGAGTTGCACGGGGCC-5′
L E L M C L N V P R

⑨VII-P30-1 Sense；5′-ATCCCCAGCACGTACGACAGGAAGACTCTGAACCACGACATCGCGCTG-3′
⑩VII-P30-2 AntiS；5′-CAGCGCGATGTCGTGGTTCAGAGTCTTCCTGTCGTACGTGCTGGGGAT-3′
VII-30のプライマーデザイン （VPGTTN→DRKTLN）
5′-ATCCCCAGCACGTACGACAGGAAGACTCTGAACCACGACATCGCGCTG-3′
3′-TAGGGGTCGTGCATGCTGTCCTTCTGAGACTTGGTGCTGTAGCGCGAC-5′
I P S T Y D R K T L N H D I A L

⑪VII-P31-1 Sense；5′-ATGACCCAGGACTGCGAAGCCTCCTACCCTGGAAAGATCACGGAGTACATG-3′
⑫VII-P31-2 AntiS；5′-CATGTACTCCGTGATCTTTCCAGGGTAGGAGGCTTCGCAGTCCTGGGTCAT-3′
VII-31のプライマーデザイン （LQQSRKVGDSPN⇒EASYPGR）
5′-ATGACCCAGGACTGCGAAGCCTCCTACCCTGGAAAGATCACGGAGTACATG-3′
3′-TACTGGGTCCTGACGCTTCGGAGGATGGGACCTTTCTAGTGCCTCATGTAC-5′
M T Q D C E A S Y P G K I T E Y M

Drawing 5



pCAGnFVII改変体発現ベクター

Drawing 6

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(71)出願人　000173555
財団法人化学及血清療法研究所
熊本県熊本市大窪一丁目６番１号
(72)発明者　副島　見事
熊本県熊本市龍田８丁目16－１　雇用促進住宅１－407
(72)発明者　水口　純
熊本県熊本市大窪１－５－35－201
(72)発明者　湯口　正人
熊本県熊本市武蔵ヶ丘７－２－17　Ｒ・Ｔ・ＴタウンＤ棟101
(72)発明者　中垣　智弘
熊本県菊池郡合志町豊岡2527－311

最終頁に続く

(54)【発明の名称】　血液凝固第ＶＩＩ因子改変体

(57)【要約】
【課題】　酵素活性を増強させた血液凝固第ＶＩＩ因子（以下、ＦＶＩＩ）及び／または活性化型血液凝固第ＶＩＩ因子（以下、ＦＶＩＩａ）の改変体を作製する。
【解決手段】　下記から選択される少なくとも一つの改変を含むことを特徴とするＦＶＩＩ／ＦＶＩＩａ改変体及び当該改変体を有効成分として含有する医薬品組成物。（ａ）ＦＶＩＩの159Ｃｙｓ-164Ｃｙｓの切断、（ｂ）ＦＶＩＩ内の、２３３番目のスレオニン（233Ｔｈｒ）から２４０番目のアスパラギン（240Ａｓｎ）のアミノ酸配列からなるループ構造を構成するアミノ酸配列またはその一部を、置換、追加または削除、（ｃ）ＦＶＩＩ内の、３０４番目のアルギニン（304Ａｒｇ）から３２９番目のシステイン（329Ｃｙｓ）の介在アミノ酸配列を構成するアミノ酸配列またはその一部を、置換、追加または削除。

【特許請求の範囲】

【請求項１】　下記から選択される少なくとも一つの改変を含むことを特徴とする血液凝固第ＶＩＩ因子（以下、ＦＶＩＩ）または活性化型血液凝固第ＶＩＩ因子（以下、ＦＶＩＩａ）の改変体。

（ａ）ＦＶＩＩ内の、１５９番目のシステイン（159Ｃｙｓ）と１６４番目のシステイン（164Ｃｙｓ）からなるジスルフィド結合（159Ｃｙｓ-164Ｃｙｓ）を切断する。

（ｂ）ＦＶＩＩ内の、２３３番目のスレオニン（233Ｔｈｒ）から２４０番目のアスパラギン（240Ａｓｎ）のアミノ酸配列からなるループ構造（以下、９９-ｌｏｏｐと称することもある）を構成するアミノ酸配列またはその一部を、置換、追加または削除する。

（ｃ）ＦＶＩＩ内の、３０４番目のアルギニン（304Ａｒｇ）から３２９番目のシステイン（329Ｃｙｓ）の介在アミノ酸配列を構成するアミノ酸配列またはその一部を、置換、追加または削除する。

【請求項２】　上記159Ｃｙｓと164ＣｙｓをＣｙｓ以外のアミノ酸残基によって置換することにより、当該159Ｃｙｓ-164Ｃｙｓが切断されることを特徴とする請求項１に記載の改変体。

【請求項３】　配列表配列番号４記載のアミノ酸配列からなる請求項１または２に記載の改変体。

【請求項４】　164ＣｙｓをＣｙｓ以外のアミノ酸残基によって置換し、かつ、２９９番目のバリン（299Ｖａｌ）をＣｙｓに置換することにより、159Ｃｙｓ-164Ｃｙｓが切断され、かつ159Ｃｙｓと299Ｃｙｓ間においてジスルフィド結合（159Ｃｙｓ-299Ｃｙｓ）が形成されることを特徴とする請求項１に記載の改変体。

【請求項５】　配列表配列番号６記載のアミノ酸配列からなる請求項１または４に記載の改変体。

【請求項６】　ＦＶＩＩの９９-ｌｏｏｐのアミノ酸配列が、他のトリプシン族セリンプロテアーゼの構造上対応するアミノ酸配列で置換されることを特徴とする請求項１に記載の改変体。

【請求項７】　他のトリプシン族セリンプロテアーゼがヒトトリプシンである請求項１または６に記載の改変体。

【請求項８】　ＦＶＩＩの９９-ｌｏｏｐ内の２３５番目のバリン（235Ｖａｌ）から２３９番目のスレオニン（239Ｔｈｒ）までのアミノ酸配列が、ヒトトリプシンの構造上対応するループ構造内にあるＡｓｐ-Ａｒｇ-Ｌｙｓ-Ｔｈｒ-Ｌｅｕで置換されることを特徴とする請求項１、６または７に記載の改変体。

【請求項９】　配列表配列番号８記載のアミノ酸配列からなる請求項１または６から８のいずれかに記載の改変体。

【請求項１０】　ＦＶＩＩ内の、３０４番目のアルギニン（304Ａｒｇ）から３２９番目のシステイン（329Ｃｙｓ）の介在アミノ酸配列を構成するアミノ酸配列またはその一部が、他のトリプシン族セリンプロテアーゼの構造上対応するアミノ酸配列で置換されることを特徴とする請求項１に記載の改変体。

【請求項１１】　他のトリプシン族セリンプロテアーゼがヒトトリプシンである請求項１または１０に記載の改変体。

【請求項１２】　ＦＶＩＩ内の、３１０番目のシステイン（310Ｃｙｓ）から３２９番目のシステイン（329Ｃｙｓ）の介在アミノ酸配列（以下、１７０-ｌｏｏｐと称することもある）を構成するアミノ酸配列またはその一部が、置換、追加または削除される請求項１、１０または１１に記載の改変体。

【請求項１３】　ＦＶＩＩの１７０-ｌｏｏｐ内の３１１番目のロイシン（311Ｌｅｕ）から３２２番目のアスパラギン（322Ａｓｎ）までのアミノ酸配列が、ヒトトリプシンの構造上対応するループ構造内にあるＧｌｕ-Ａｌａ-Ｓｅｒ-Ｔｙｒ-Ｐｒｏ-Ｇｌｙ-Ｌｙｓで置換されることを特徴とする請求項１または１０から１２のいずれかに記載の改変体。

【請求項１４】　配列表配列番号１０記載のアミノ酸配列からなる請求項１または１０から１３のいずれかに記載の改変体。

【請求項１５】　ＦＶＩＩの９９-ｌｏｏｐ内の２３５番目のバリン（235Ｖａｌ）から２３９番目のスレオニン（239Ｔｈｒ）までのアミノ酸配列が、ヒトトリプシンの構造上対応するループ構造内にあるＡｓｐ-Ａｒｇ-Ｌｙｓ-Ｔｈｒ-Ｌｅｕで置換され、かつ、１７０-ｌｏｏｐ内の３１１番目のロイシン（311Ｌｅｕ）から３２２番目のアスパラギン（322Ａｓｎ）までのアミノ酸配列が、ヒトトリプシンの構造上対応するループ構造内にあるＧｌｕ-Ａｌａ-Ｓｅｒ-Ｔｙｒ-Ｐｒｏ-Ｇｌｙ-Ｌｙｓで置換されることを特徴とする請求項１に記載の改変体。

【請求項１６】　配列表配列番号１２記載のアミノ酸配列からなる請求項１または１５に記載の改変体。

【請求項１７】　請求項１から１６のいずれかに記載の改変体を有効成分として含有する医薬品組成物。

【請求項１８】　請求項１７の医薬品組成物からなる血友病インヒビター患者の治療に有効な薬剤。

【発明の詳細な説明】

【０００１】

【発明の属する技術分野】本願発明は、酵素活性を増強させた血液凝固第ＶＩＩ因子（以下、ＦＶＩＩと称することがある）及び／または活性化血液凝固第ＶＩＩ因子（以下、ＦＶＩＩａと称することがある）の改変体に関するものである。詳細には、本願発明は、ＦＶＩＩに特有なアミノ酸配列を、置換・欠損することにより、活性が増強されたＦＶＩＩ／ＦＶＩＩａ改変体、当該改変体を有効成分として含有する医薬品組成物、及び当該医薬

品組成物からなる血友病インヒビター患者の治療に有効な薬剤に関するものである。

【０００２】

【従来の技術および解決すべき課題】ＦＶＩＩはビタミンＫ依存性の血液凝固因子であり、外因系血液凝固の開始因子であることは広く知られている。他のビタミンＫ依存性凝固因子と同様にＮ末端から３５残基までのアミノ酸配列に１０個のγカルボキシグルタミン酸（以下、Ｇｌａと称することがある）からなるＧｌａ領域を有している（Ｐｒｏｃ.Ｎａｔｌ.Ａｃａｄ.Ｓｃｉ.ＵＳＡ, ｖｏｌ.８３, ｐ.２４１２-２４１６, １９８６）。ＦＶＩＩは、ｉｎ ｖｉｔｒｏにおいて、活性化血液凝固第Ｘ因子（以下、ＦＸaと称することがある）、活性化血液凝固第ＩＸ因子（以下、ＦＩＸaと称することがある）またはトロンビン（以下、ＦＩＩaと称することがある）によって、152Ａｒｇ-153Ｉｌｅが加水分解され、一個のＳ-Ｓ結合で架橋されたＨ鎖とＬ鎖から構成される活性型ＦＶＩＩすなわちＦＶＩＩaに変換されることは知られている（Ｊ.Ｂｉｏｌ.Ｃｈｅｍ., ｖｏｌ.２５１, ｐ.４７９７-４８０２, １９７６）。

【０００３】ＦＶＩＩａ自体の酵素活性は極めて弱く、補酵素である組織因子（ＴＦ）と結合すると劇的に上昇する（Komiyama et al., Biochemistry, 29(40), pp.9418-25(1990)）。ＦＶＩＩａとＴＦの１次構造、その複合体の結晶構造、さらに両分子間の結合部位もアミノ酸残基レベルで判明しているが、その触媒活性増幅機構の詳細（ＴＦ結合に伴う立体構造変化）は依然として不明である（Banner et al., , et al., Nature 380(6569): pp.41-6(1996)）。

【０００４】血友病Ａ及び血友病Ｂ患者に対する補充療法として、血液凝固第ＶＩＩＩ因子（以下、ＦＶＩＩＩと称することがある）及び血液凝固第ＩＸ因子（以下、ＦＩＸと称することがある）製剤の投与が行なわれている。しかし、当該治療法に伴いＦＶＩＩＩ及びＦＩＸに対する中和抗体（インヒビターと呼ばれることもある）の出現が問題視されている。

【０００５】このようなインヒビターを生じた血友病患者の対処療法として、(1)ＦＶIII因子の過剰投与、(2)ブタＦＶIII因子の投与、(3)ＦＩＩ、ＦＶＩＩ、ＦＩＸ及びＦＸからなる複合体製剤の投与、(4)ＦＶＩＩａ製剤の投与などがある。しかしながらこれらの方法は、それぞれ(1)については、より高力価なインヒビターの誘導による症状悪化、(2)については、抗原性によるショック、(3)については、血栓・ＤＩＣの誘発、(4)については、治療効果が不十分であることや大量・頻回投与によりコストが高いなどの問題を抱えている。これらの中で、効果と危険性のバランスを考慮した場合、最も効率的なものは(4)のＦＶＩＩａ製剤の投与である。しかしながら、ＦＶＩＩａ製剤はその活性の弱さのため、止血効果を発揮するには、前述したように大量投与と頻回投与を必要とし、治療コストを大きく高めている。また、その治療効果も血友病患者に対して行われている従来の補充療法に比べれば充分とはいえない。

【０００６】この問題を解決するための手段として、酵素活性を上昇させたＦＶＩＩの改変体を作製することが挙げられるが、これは一般的に困難であることが知られている（タンパク質の構造入門、勝部幸輝ら監修、教育社発行、1992年）。特に、血液凝固因子について、以下の理由により改変による活性増強は困難と考えられている。

【０００７】血友病は血液凝固因子の異常であるが、量的欠損に伴う活性低下と質的異常による活性低下の2つに分類される。このうち質的異常の多くは（ポイント）ミューテーションであることが知られており、ＦＩＸの異常である血友病Ｂの患者の解析が行われた結果、ＦＩＸの構造全域にわたって分子異常が存在することが明らかとなり、中にはたった１個のアミノ酸が置換されただけで、活性が１％以下になる例が多数ある。従って、血液凝固因子についてむやみに改変を行っても、活性低下を招くのは明らかである。

【０００８】また、Alanine Scannningで得られた情報（Dickinson et al., Proc. Natl. Acad. Sci. USA, 93(25), pp.14379-84(1996)）によれば、ＦＶＩＩの１１２個のAlanine置換体ついて、その中で酵素活性が上がったものは唯一１つであり、しかもその規則性は見いだされていない。

【０００９】その他の試みとして、Hopfnerらは、ＦＩＸを構成する一部のドメインの数アミノ酸残基から構成される構造単位を欠損・置換する方法を用い、合成基質活性を上昇させたＦＩＸフラグメント改変体を作製した（EMBO J, 16(22), pp.6626-35(1997)）。しかしながら、これはインタクトなＦＩＸではなく、ＦＩＸの部分フラグメントを大腸菌で発現させたもので合成基質活性を見ているに過ぎないため、血液凝固活性を増強しうるどころか血液凝固活性すら有さないものである。さらに、これはＦＩＸに関するものであり、構造も特異性も全く異なる別物質であるＦＶＩＩに対して何ら示唆するものではなく、ＦＶＩＩの酵素活性を増強させた改変体についても、これまで何ら報告はない。

【００１０】このように、強い酵素活性を有する改変体の作製は、特に血液凝固因子においては困難と考えられていた。ＦＩＸにおいて、その部分フラグメントについて合成基質活性を上げる試みはなされたものの、インタクトな分子として高い酵素活性を有する血液凝固因子の改変体についてはこれまでも報告例はない。

【００１１】従って、本発明の解決すべき課題は、一般に血液凝固因子の改変は困難と考えられている状況において、血友病インヒビター患者の治療に有効な強い活性を有するＦＶＩＩ及び／またはＦＶＩＩａを作製・提供することである。

【００１２】
【課題を解決するための手段】上記のような状況において、本発明者らは、それ自身高い酵素活性を有するＦＶＩＩを作製すべく鋭意研究を重ね種々の検討を行った結果、本願発明を完成するに至った。本願発明は、ＦＶＩＩと各種セリンプロテアーゼとアミノ酸配列構造を比較し、ＦＶＩＩに特有のアミノ酸配列部位を明確にし、その特有な部位を、欠損・置換することにより、活性が増強されたＦＶＩＩ及び／またはＦＶＩＩａ改変体を作製することに成功したものである。
【００１３】
【発明の構成】トリプシン族に類する一群のセリンプロテアーゼの基本構造は、約２５０残基からなり、アミノ酸配列上でおよそ、その前半と後半の２つのドメインに分けられる（図１）。各ドメイン内にはそれぞれ６本のβストランドがあり、プロテアーゼとして計１２本のβストランドを有する構造で形成されている（図２）。これら１２本のβストランドはいわばセリンプロテアーゼの骨格構造となっており、各ストランド間をつなぐループないし、ヘリックス領域が、その基質特異性やコファクターとの反応性などのプロテアーゼ活性を担っていると考えられている。セリンプロテアーゼの例としては、ＦＩＩ、ＦＶＩＩ、ＦVIII、ＦＩＸ、ＦＸ等の各種血液凝固因子、プラスミン等の血栓溶解酵素、またはトリプシン、キモトリプシン、エラスターゼなどの消化酵素がある。そこで、ＦＶＩＩをはじめとする各種セリンプロテア--ゼのアミノ酸配列構造の比較を行い、ＦＶＩＩに特徴的な領域を特定した（図３）。そして、これらの部位を改変のターゲットとし、他のセリンプロテアーゼの構造を参考に、ＦＶＩＩのアミノ酸配列を欠損・置換することによって、高い酵素活性を有するＦＶＩＩ改変体を作製した。これらの改変体について詳細に説明する。
【００１４】（ａ）159Ｃｙｓ-164Ｃｙｓ結合が切断された改変体
（ａ-①）159ＣｙｓとＣｙｓ164ＣｙｓをＣｙｓ以外のアミノ酸残基によって置換することにより、当該159Ｃｙｓ-164Ｃｙｓが切断された改変体（ＶＩ１-５）。この改変体の具体例として、Ｃｙｓをそれぞれアラニン（Ａｌａ）に置換したものを配列表配列番号３または４に記載した。ここで、置換に用いるＣｙｓ以外のアミノ酸残基の一例として、Ａｌａを選択したが、置換によって、Ｃｙｓ-Ｃｙｓ結合を切断すること以外に酵素活性を失活させるなどの大きな障害を与えない限り、任意のアミノ酸が選択可能である。
【００１５】（ａ-②）164ＣｙｓをＣｙｓ以外のアミノ酸残基によって置換し、かつ、２９９番目のヴァリン（299Ｖａｌ）をＣｙｓに置換することにより、159Ｃｙｓ-164Ｃｙｓが切断され、かつ159ＣｙｓとＣｙｓ間においてジスルフィド結合（159Ｃｙｓ-299Ｃｙｓ）が形成された改変体（ＶＩＩ-６）。この改変体の具体例として、Ｃｙｓ以外のアミノ酸残基としてＡｌａを用いて置換したものを配列表配列番号５または６に記載した。ここで、置換に用いるＣｙｓ以外のアミノ酸残基については上述の通り、置換によって159Ｃｙｓ-164Ｃｙｓ結合を切断すること以外に酵素活性を失活させるなどの大きな障害を与えない限り、Ａｌａ以外の他のアミノ酸が選択可能である。
【００１６】（ｂ）ＦＶＩＩ内の、２３３番目のスレオニン（233Ｔｈｒ）から２４０番目のアスパラギン（240Ａｓｎ）のアミノ酸配列からなるループ構造（以下、９９-ｌｏｏｐと称することもある）を構成するアミノ酸配列またはその一部が、置換、追加または削除された改変体。
この領域は、図３に示すようにセリンプロテアーゼに共通に存在するβストランド５とβストランド６の間に介在するアミノ酸配列を含むものである。この領域を他のトリプシン族セリンプロテアーゼの構造上対応するアミノ酸配列で置換することが好ましい。トリプシン族セリンプロテアーゼの好適な一例として、ヒトトリプシンが挙げられる。さらに、具体的な例として、ＦＶＩＩの９９-ｌｏｏｐ内の２３５番目のバリン（235Ｖａｌ）から２３９番目のスレオニン（239Ｔｈｒ）までのアミノ酸配列が、トリプシンのループ構造内にあるＡｓｐ-Ａｒｇ-Ｌｙｓ-Ｔｈｒ-Ｌｅｕで置換された改変体（ＶＩＩ-３０）が挙げられる。この改変体を配列表配列番号７または８に記載した。
【００１７】（ｃ）ＦＶＩＩ内の、３０４番目のアルギニン（304Ａｒｇ）から３２９番目のシステイン（329Ｃｙｓ）の介在アミノ酸配列を構成するアミノ酸配列またはその一部が、置換、追加または削除された改変体。
特にこの領域は、図３に示すように、セリンプロテアーゼに共通に存在するβストランド８とβストランド９の間に介在するアミノ酸配列において、ＦＶＩＩは他のセリンプロテアーゼと比較して数アミノ酸残基長いという特徴を有することから、ＦＶＩＩ改変における好適なターゲットとなりうるものと推測される。この領域を、他のトリプシン族セリンプロテアーゼの構造上対応するアミノ酸配列で置換することが好ましい。トリプシン族セリンプロテアーゼの好適な一例として、ヒトトリプシンが挙げられる。また、ＦＶＩＩ内の置換、追加、削除しうる好ましい領域は、３１０番目のシステイン（310Ｃｙｓ）から３２９番目のシステイン（329Ｃｙｓ）のアミノ酸配列からなるループ構造（１７０-ｌｏｏｐと称することもある）を構成するアミノ酸配列またはその一部である。さらに、具体的な例として、ＦＶＩＩの１７０-ｌｏｏｐ内の３１１番目のロイシン（311Ｌｅｕ）から３２２番目のアスパラギン（322Ａｓｎ）までのアミノ酸配列が、ヒトトリプシンのループ構造内にあるＧｌｕ-Ａｌａ-Ｓｅｒ-Ｔｙｒ-Ｐｒｏ-Ｇｌｙ-Ｌｙ

ｓで置換された改変体（ＶＩＩ-３１）が挙げられる。この改変体を配列表配列番号９または１０に記載した。

【００１８】さらに、上記（ａ）から（ｃ）の改変を適宜組み合わせることも可能である。その具体例として、例えば、（ｂ）と（ｃ）の組み合わせ、すなわち、ＦＶＩＩの９９-ｌｏｏｐ内の２３５番目のバリン（235Ｖａｌ）から２３９番目のスレオニン（239Ｔｈｒ）までのアミノ酸配列が、ヒトトリプシンのループ構造内にあるＡｓｐ-Ａｒｇ-Ｌｙｓ-Ｔｈｒ-Ｌｅｕで置換され、かつ、１７０-ｌｏｏｐ内の３１１番目のロイシン（311Ｌｅｕ）から３２２番目のアスパラギン（322Ａｓｎ）までのアミノ酸配列が、トリプシンのループ構造内にあるＧｌｕ-Ａｌａ-Ｓｅｒ-Ｔｙｒ-Ｐｒｏ-Ｇｌｙ-Ｌｙｓで置換された改変体（ＶＩＩ-３９）が挙げられる。この改変体を配列表配列番号１１または１２に記載した。

【００１９】上述した改変体は、遺伝子組換え法を用いて得ることができる。発現宿主としては、動物細胞等の真核細胞が好ましい。本発明の改変体は、上記各改変体のアミノ酸配列をコードするｃＤＮＡを適当な発現ベクターに組み込み、宿主細胞にトランスフェクトし、目的の遺伝子を発現している細胞をクローニングし、得られた安定発現株を培養後、精製することにより得られる。

【００２０】本願発明のＦＶＩＩ改変体は各種化学処理等を行い、活性化型ＦＶＩＩ（ＦＶＩＩａ）改変体として使用することができる。

【００２１】本願発明のＦＶＩＩ／ＦＶＩＩａ改変体は、治療、診断または他の用途のために製薬学的調合剤に処方することができる。静脈内投与のための調合剤に対しては、組成物を、通常、生理学的に適合しうる物質、例えば塩化ナトリウム、グリシン等を含み、かつ生理学的条件に適合しうる緩衝されたｐＨを有する水溶液中に溶解する。また、長期安定性の確保の観点から、最終的剤型として凍結乾燥製剤の形態をとることも考慮されうる。なお、静脈内に投与される組成物のガイドラインは政府の規則、例えば「生物学的製剤基準」によって確立されている。本願発明のＦＶＩＩ／ＦＶＩＩａ改変体からなる医薬品組成物の具体的な用途としては、ＦＶＩＩＩまたはＦＩＸの補充療法により当該血液凝固因子に対してインヒビターを生じた血友病インヒビター患者の治療が挙げられる。

【００２２】

【実施例】本願発明を実施例により例示するが、これら実施例は本願発明を限定するものではない。本願発明について添付図面を参照して特定な実施例にて例示する。実施例は改変体を動物細胞（CHO-K1）の培養上清中に発現させたものである。以下特に断りが無い限り、遺伝子組換えに関わる試薬等は、宝酒造、東洋紡、パーキンエルマーアプライドNew England Biolabs 社の製品を用いた。

【００２３】《実施例１．ＦＶＩＩｃＤＮＡのクローニング》ヒト肝臓ｃＤＮＡライブラリー（宝酒造）を購入し、文献等（Molecular Basis of Thrombosia and Hemostasis）で公知のｃＤＮＡ配列（配列表配列番号１に記載）を基にSalIサイトを付加したＦＶＩＩ合成ＤＮＡセンスプライマー（VII-PWN；GGGGTCGACATGGTCTCCCAGGCCCTCAGGCTCCTCTGCCTTCTG）及び、BamHIサイトを付加したアンチセンスプライマー（VII-PWC；CCCGGATCCCTAGGGAAATGGGGCTCGCAGGAGGACTCCTGGGCG）を用いてＰＣＲを行い、市販のクローニングベクターpCRII（Invitrogen社）にクローニングした。この際、常法によりＤＮＡシークエンスを行い、文献等で公知の配列（Hagen FS et al , PNAS 1986; 83; 2412-6）を有することを確認した。

【００２４】《実施例２．ＦＶＩＩ発現ベクターの調製》発現ベクターpCAGn（特許第２８２４４３４号公報）をSalI、BamHIで消化し、そこにＦＶＩＩをコードした配列を含む上記実施例１で調製したＤＮＡフラグメントをSalI、BamHIでカットしたものをライゲーションし、大腸菌JM105に形質転換し、アンピシリン含有のＬＢ寒天培地上で培養し、形質転換大腸菌を選択した。出現したコロニーを市販の培地で一晩培養し、目的の発現プラスミドを抽出精製し「pVII-W」を調製した。この発現ベクターのＤＮＡシークエンスを行い、目的の遺伝子配列を有することを確認した。

【００２５】《実施例３．改変体発現ベクターの調製》図４に示すアミノ酸配列を有する各ＦＶＩＩ改変体を、以下の方法で作成した。なお図４は、ＦＶＩＩの１５３番目のイソロイシンよりＣ末側のアミノ酸配列のみ示したもので、１５２番目のアルギニンよりＮ末側のアミノ酸については、いずれも改変は行っておらず野生型と同じである。図５に示す合成ＤＮＡプライマーを用いてＦＶＩＩ遺伝子を鋳型としてＰＣＲを行いそれぞれの増幅断片を得る。各増幅断片と、発現ベクターpCAGnをSalI及びBamHIでカットしたものをライゲーションし、大腸菌JM105に形質転換し、アンピシリン含有のＬＢ寒天培地上で培養し、形質転換大腸菌を選択した。出現したコロニーを市販の培地で一晩培養し、目的の発現プラスミドを抽出精製し「pVII-5」、「pVII-30」、及び「pVII-31」を調製した（図６）。また、「pVII-6」については、図５に記載のプライマー⑤及び⑥を用いて得られた遺伝子を鋳型にし、さらにプライマー⑦及び⑧を用いてＰＣＲを行うことにより得られた。また、「pVII-39」については、プライマー⑨及び(10)を用いて得られた遺伝子を鋳型にし、さらにプライマー(11)及び(12)を用いてＰＣＲを行うことにより得られた。さらにＤＮＡシークエンスを行い、これらのプラスミドが目的の配列を有することを確認した。

【００２６】《実施例４．各改変体の培養上清への発現及び精製》上記発現ベクターを、市販のリポフェクチン

試薬でＣＨＯ細胞に対して形質導入を行い、G418(１mg/ml)で選択し、目的の遺伝子を発現している細胞を限外希釈法によりクローニングした。ＦＶＩＩ改変体の発現の確認は、市販のＦＶＩＩに対するＥＬＩＳＡキット（アセラクロムＦＶＩＩ；Diagnostica Strago社）で行った。得られた安定発現株を無血清培地（ASF104、味の素、ペニシリン、ストレプトマイシン、20μg/mlビタミンK、１mM酪酸）で培養し、抗ヒトＦＶＩＩモノクローナル抗体カラムで精製した（特許第２８２４４３０号公報）。平衡化・洗浄及び溶出は、平衡化・洗浄バッファー（50mM Tris, pH 7.2, 0.1M NaCl, 50mM Benzamidine-HCl, ２mM Ca^{2+}）、溶出バッファー（50mM Tris, pH 7.2, 0.1MNaCl, 50mM Benzamidine-HCl, 10mM EDTA）を用いて行った。純化された改変体をＳＤＳ-ＰＡＧＥ、または市販のＦＶＩＩに対する抗体を用いて、ウエスタンブロットを行い、ＦＶＩＩ改変体であることを確認した。

【００２７】《実施例５．各改変体の凝固活性の測定》各改変体の凝固活性は常法に従い、ＦＶＩＩ欠乏血漿を用いた凝固法で測定した。精製した各改変体を50～５ng/mlになるようにTris-BSAで希釈し、ＦＶＩＩ欠乏血症と等量混ぜ、37℃で３分加温後、再脂質化ＴＦ（トロンボプラスチン；Dade社）を等量添加し、凝固反応を開始させた。凝固時間を測定し、標準曲線と希釈率より凝固活性を求めた。凝固活性を蛋白濃度（Bradford法で測定）当たりに換算し比活性を求めた結果を表１に記す。その結果、本発明のＦＶＩＩ改変体は、血漿由来ＦＶＩＩ及び野生型組換えＦＶＩＩと比較して、２～６倍高い凝固活性を有することが明らかとなった。

【００２８】

【表１】

サンプル	改変内容	凝固活性 U/ml	蛋白濃度 μg/ml	比活性 U/ml	相対比 %
血漿由来	天然品	2,000	1,000	2,000	100
VII-W	組換え野生型	3,400	1,700	2,000	100
VII-5	159Cys-164Cys の切断	4,954	1,032	4,800	240
VII-6	159Cys-299Cys の形成	6,636	1,293	5,132	257
VII-30	loop99 を Trypsin 型へ	3,361	685	4,907	245
VII-31	loop170 を Trypsin 型へ	3,589	877	4,093	205
VII-39	loop99+170 を Trypsin 型へ	8,954	773	11,584	579

【００２９】《実施例６．活性化された各改変体の調製》精製した各改変体を、50mM Tris, pH 7.45, 0.1M NaClに透析し、ＦＸａを1/100（モル比）加え、50mM Tris, pH 7.45, 0.1M NaCl, 0.1% PEG 8000, 100μg/mLリン脂質（Platerin(登録商標) Organotecnica社）、10mM Ca^{2+}、37℃の条件下、１～60分でインキュベーションし活性化した。活性化後、50mM Benzamidine-HClを加えて反応を停止し、抗ヒトＦＶＩＩモノクローナル抗体カラムで精製した（実施例４と同じ方法）。精製済みの各活性化改変体はTBS pH 8.0 (0.1% PEG 8000含有)に透析し、-80℃に凍結保存した。活性化の程度は、ＳＤＳ-ＰＡＧＥで確認した。

【００３０】《実施例７．活性化された各改変体の合成基質に対する水解活性測定》実施例６に従い活性化された改変体ＶＩＩａ-３１を0.1μMになるまで50mMTris-HCl, 100mM NaCl, 10mM Ca^{2+}, 0.1% PEG 8000, pH 8.0で希釈し、そこに種々の合成基質を最終濃度1.0mMになるように加え、最終容量を200μｌとし、30℃で反応させ、１分間当たりの基質の水解量を見た。温度制御が可能な microplate reader Spectra max plus (Molecular device社)でpNAの遊離を405nmによる発色度として測定した。この結果を表２に示す。本発明の改変体の一つであるＶＩＩａ-３１は、何れの合成基質を対しても野生型（ＶＩＩａ-W）より高い水解活性を示し、その範囲は２～２３倍であった。

【００３１】

【表２】

基質名	構造	水解活性／mOD_{405nm}/min VIIa-W	VIIa-31	比 31/W
Chromozym tPA	D-Phe-Gly-Arg	37.6	117.6	3
S-2288	H-D-Ile-Pro-Arg	25.8	304.2	12
S-2366	pyro-Glu-Pro-Arg	11.5	267.8	23
S-2238	H-D-Phe-Pip-Arg	11.3	86.6	8
Chromozym X	D-Nile-Gly-Arg	13.0	48.6	4
S-2302	H-D-Pro-Phe-Arg	7.4	38.3	5
S-2765	Z-D-Arg-Gly-Arg	13.3	20.6	2
Chromozym TRY	CBz-Val-Gly-Arg	5.6	28.2	5
S-2444	pyro-Glu-Gly-Arg	1.5	18.9	13
S-2222	Bz-Ile-Glu-Gly-Arg	5.6	16.4	3
S-2403	pyro-Glu-Phe-Lys	0.3	5.9	19

【００３２】

【発明の効果】このように本願発明により得られたＦＶ

ＩＩ及び／またはＦＶＩＩａの改変体は、野生型のＦＶＩＩに比べて明らかに高い酵素活性を有するものである。従って、本願発明の改変体は、血友病インヒビター患者への補充療法として極めて有効な薬剤となりうるものである。

【配列表】

SEQUENCE LISTING

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TGC AAG GAG GAG CAG TGC TCC TTC GAG GAG GCC CGG GAG ATC TTC AAG     96
Cys Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys
            20                  25                  30
GAC GCG GAG AGG ACG AAG CTG TTC TGG ATT TCT TAC AGT GAT GGG GAC    144
Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp
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Gln Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln
    50                  55                  60
CTC CAG TCC TAT ATC TGC TTC TGC CTC CCT GCC TTC GAG GGC CGG AAC    240
Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn
65                  70                  75                  80
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            100                 105                 110
CGG TGC CAC GAG GGG TAC TCT CTG CTG GCA GAC GGG GTG TCC TGC ACA    384
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145                 150                 155                 160
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His Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu
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225                 230                 235                 240
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His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp
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CAT GTG GTG CCC CTC TGC CTG CCC GAA CGG ACG TTC TCT GAG AGG ACG    816
His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr
            260                 265                 270
CTG GCC TTC GTG CGC TTC TCA TTG GTC AGC GGC TGG GGC CAG CTG CTG    864
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Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn
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His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr
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Lys Asp Ser Cys Lys Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr
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Ala Thr Val Gly His Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile
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TGC AAG GAG GAG CAG TGC TCC TTC GAG GAG GCC CGG GAG ATC TTC AAG     96
Cys Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys
            20                  25                  30
GAC GCG GAG AGG ACG AAG CTG TTC TGG ATT TCT TAC AGT GAT GGG GAC    144
Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp
        35                  40                  45
CAG TGT GCC TCA AGT CCA TGC CAG AAT GGG GGC TCC TGC AAG GAC CAG    192
Gln Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln
    50                  55                  60
CTC CAG TCC TAT ATC TGC TTC TGC CTC CCT GCC TTC GAG GGC CGG AAC    240
Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn
65                  70                  75                  80
TGT GAG ACG CAC AAG GAT GAC CAG CTG ATC TGT GTG AAC GAG AAC GGC    288
Cys Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly
                85                  90                  95
GGC TGT GAG CAG TAC TGC AGT GAC CAC ACG GGC ACC AAG CGC TCC TGT    336
Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys
            100                 105                 110
CGG TGC CAC GAG GGG TAC TCT CTG CTG GCA GAC GGG GTG TCC TGC ACA    384
Arg Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr
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Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg
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AAT GCC AGC AAA CCC CAA GGC CGA ATT GTG GGG GGC AAG GTG GCC CCC    480
Asn Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Ala Pro
145                 150                 155                 160
AAA GGG GAG GCC CCA TGG CAG GTC CTG TTG TTG GTG AAT GGA GCT CAG    528
Lys Gly Glu Ala Pro Trp Gln Val Leu Leu Leu Val Asn Gly Ala Gln
                165                 170                 175
TTG TGT GGG GGG ACC CTG ATC AAC ACC ATC TGG GTG GTC TCC GCG GCC    576
Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala
            180                 185                 190
CAC TGT TTC GAC AAA ATC AAG AAC TGG AGG AAC CTG ATC GCG GTG CTG    624
His Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu
        195                 200                 205
GGC GAG CAC GAC CTC AGC GAG CAC GAC GGG GAT GAG CAG AGC CGG CGG    672
Gly Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg
    210                 215                 220
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Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn
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His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr
260 265 270
CTG GCC TTC GTG CGC TTC TCA TTG GTC AGC GGC TGG GGC CAG CTG CTG 864
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GAG TGG CTG CAA AAG CTC ATG CGC TCA GAG CCA CGC CCA GGA GTC CTC 1200
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35 40 45
Gln Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln

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     50                  55                  60
Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn
 65                  70                  75                  80
Cys Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly
                 85                  90                  95
Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys
            100                 105                 110
Arg Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr
        115                 120                 125
Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg
    130                 135                 140
Asn Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Ala Pro
145                 150                 155                 160
Lys Gly Glu Ala Pro Trp Gln Val Leu Leu Leu Val Asn Gly Ala Gln
                165                 170                 175
Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala
            180                 185                 190
His Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu
        195                 200                 205
Gly Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg
    210                 215                 220
Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn
225                 230                 235                 240
His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp
                245                 250                 255
His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr
            260                 265                 270
Leu Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu
        275                 280                 285
Asp Arg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg
    290                 295                 300
Leu Met Thr Gln Asp Cys Leu Gln Gln Ser Arg Lys Val Gly Asp Ser
305                 310                 315                 320
Pro Asn Ile Thr Glu Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser
                325                 330                 335
Lys Asp Ser Cys Lys Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr
            340                 345                 350
Arg Gly Thr Trp Tyr Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys
        355                 360                 365
Ala Thr Val Gly His Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile
    370                 375                 380
Glu Trp Leu Gln Lys Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu
385                 390                 395                 400
Leu Arg Ala Pro Phe Pro
                405
<210> 5
<211> 1221
<212> DNA
<213> artificial sequence
<220>
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<223> Amino acid sequence of recombinant mutant of blood coagulation factor VII in which the 164th Cysteine is replaced with Alanine and the 299 Valine is replaced with Cysteine, and cDNA sequence coding thereof.

<400> 5

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GCC AAC GCG TTC CTG GAG GAG CTG CGG CCG GGC TCC CTG GAG AGG GAG     48
Ala Asn Ala Phe Leu Glu Glu Leu Arg Pro Gly Ser Leu Glu Arg Glu
  1               5                  10                  15

TGC AAG GAG GAG CAG TGC TCC TTC GAG GAG GCC CGG GAG ATC TTC AAG     96
Cys Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys
             20                  25                  30

GAC GCG GAG AGG ACG AAG CTG TTC TGG ATT TCT TAC AGT GAT GGG GAC    144
Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp
         35                  40                  45

CAG TGT GCC TCA AGT CCA TGC CAG AAT GGG GGC TCC TGC AAG GAC CAG    192
Gln Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln
     50                  55                  60

CTC CAG TCC TAT ATC TGC TTC TGC CTC CCT GCC TTC GAG GGC CGG AAC    240
Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn
 65                  70                  75                  80

TGT GAG ACG CAC AAG GAT GAC CAG CTG ATC TGT GTG AAC GAG AAC GGC    288
Cys Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly
                 85                  90                  95

GGC TGT GAG CAG TAC TGC AGT GAC CAC ACG GGC ACC AAG CGC TCC TGT    336
Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys
            100                 105                 110

CGG TGC CAC GAG GGG TAC TCT CTG CTG GCA GAC GGG GTG TCC TGC ACA    384
Arg Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr
        115                 120                 125

CCC ACA GTT GAA TAT CCA TGT GGA AAA ATA CCT ATT CTA GAA AAA AGA    432
Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg
    130                 135                 140

AAT GCC AGC AAA CCC CAA GGC CGA ATT GTG GGG GGC AAG GTG TGC CCC    480
Asn Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Cys Pro
145                 150                 155                 160

AAA GGG GAG GCC CCA TGG CAG GTC CTG TTG TTG GTG AAT GGA GCT CAG    528
Lys Gly Glu Ala Pro Trp Gln Val Leu Leu Leu Val Asn Gly Ala Gln
                165                 170                 175

TTG TGT GGG GGG ACC CTG ATC AAC ACC ATC TGG GTG GTC TCC GCG GCC    576
Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala
            180                 185                 190

CAC TGT TTC GAC AAA ATC AAG AAC TGG AGG AAC CTG ATC GCG GTG CTG    624
His Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu
        195                 200                 205

GGC GAG CAC GAC CTC AGC GAG CAC GAC GGG GAT GAG CAG AGC CGG CGG    672
Gly Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg
    210                 215                 220

GTG GCG CAG GTC ATC ATC CCC AGC ACG TAC GTC CCG GGC ACC ACC AAC    720
Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn
225                 230                 235                 240

CAC GAC ATC GCG CTG CTC CGC CTG CAC CAG CCC GTG GTC CTC ACT GAC    768
```

```
His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp
                245                 250                 255
CAT GTG GTG CCC CTC TGC CTG CCC GAA CGG ACG TTC TCT GAG AGG ACG   816
His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr
            260                 265                 270
CTG GCC TTC GTG CGC TTC TCA TTG GTC AGC GGC TGG GGC CAG CTG CTG   864
Leu Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu
        275                 280                 285
GAC CGT GGC GCC ACG GCC CTG GAG CTC ATG TGC CTC AAC GTG CCC CGG   912
Asp Arg Gly Ala Thr Ala Leu Glu Leu Met Cys Leu Asn Val Pro Arg
    290                 295                 300
CTG ATG ACC CAG GAC TGC CTG CAG CAG TCA CGG AAG GTG GGA GAC TCC   960
Leu Met Thr Gln Asp Cys Leu Gln Gln Ser Arg Lys Val Gly Asp Ser
305                 310                 315                 320
CCA AAT ATC ACG GAG TAC ATG TTC TGT GCC GGC TAC TCG GAT GGC AGC  1008
Pro Asn Ile Thr Glu Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser
                325                 330                 335
AAG GAC TCC TGC AAG GGG GAC AGT GGA GGC CCA CAT GCC ACC CAC TAC  1056
Lys Asp Ser Cys Lys Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr
            340                 345                 350
CGG GGC ACG TGG TAC CTG ACG GGC ATC GTC AGC TGG GGC CAG GGC TGC  1104
Arg Gly Thr Trp Tyr Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys
        355                 360                 365
GCA ACC GTG GGC CAC TTT GGG GTG TAC ACC AGG GTC TCC CAG TAC ATC  1152
Ala Thr Val Gly His Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile
    370                 375                 380
GAG TGG CTG CAA AAG CTC ATG CGC TCA GAG CCA CGC CCA GGA GTC CTC  1200
Glu Trp Leu Gln Lys Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu
385                 390                 395                 400
CTG CGA GCC CCA TTT CCC TAG                                     1221
Leu Arg Ala Pro Phe Pro
                405
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<210> 6
<211> 406
<212> PRT
<213> artificial sequence
<220>
<223> Amino acid sequence of recombinant mutant of blood coagulation factor VII in which the 164th Cysteine is replaced with Alanine and the 299 Valine is replaced with Cysteine.
<400> 6

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Ala Asn Ala Phe Leu Glu Glu Leu Arg Pro Gly Ser Leu Glu Arg Glu
  1               5                  10                  15
Cys Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys
            20                  25                  30
Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp
        35                  40                  45
Gln Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln
    50                  55                  60
Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn
```

65 70 75 80
Cys Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly
85 90 95
Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys
100 105 110
Arg Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr
115 120 125
Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg
130 135 140
Asn Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Cys Pro
145 150 155 160
Lys Gly Glu Ala Pro Trp Gln Val Leu Leu Leu Val Asn Gly Ala Gln
165 170 175
Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala
180 185 190
His Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu
195 200 205
Gly Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg
210 215 220
Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn
225 230 235 240
His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp
245 250 255
His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr
260 265 270
Leu Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu
275 280 285
Asp Arg Gly Ala Thr Ala Leu Glu Leu Met Cys Leu Asn Val Pro Arg
290 295 300
Leu Met Thr Gln Asp Cys Leu Gln Gln Ser Arg Lys Val Gly Asp Ser
305 310 315 320
Pro Asn Ile Thr Glu Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser
325 330 335
Lys Asp Ser Cys Lys Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr
340 345 350
Arg Gly Thr Trp Tyr Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys
355 360 365
Ala Thr Val Gly His Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile
370 375 380
Glu Trp Leu Gln Lys Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu
385 390 395 400
Leu Arg Ala Pro Phe Pro
405

<210> 7
<211> 1221
<212> DNA
<213> artificial sequence
<220>
<223> Amino acid sequence of recombinant mutant of blood coagulation fac

tor VII in which the 5 amino acid residues from the 235th Valine to 239th threonine are replaced with Asp-Arg-Lys-Thr-Leu, and cDNA sequence coding thereof.

<400> 7

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GCC AAC GCG TTC CTG GAG GAG CTG CGG CCG GGC TCC CTG GAG AGG GAG     48
Ala Asn Ala Phe Leu Glu Glu Leu Arg Pro Gly Ser Leu Glu Arg Glu
 1               5                  10                  15
TGC AAG GAG GAG CAG TGC TCC TTC GAG GAG GCC CGG GAG ATC TTC AAG     96
Cys Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys
            20                  25                  30
GAC GCG GAG AGG ACG AAG CTG TTC TGG ATT TCT TAC AGT GAT GGG GAC    144
Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp
        35                  40                  45
CAG TGT GCC TCA AGT CCA TGC CAG AAT GGG GGC TCC TGC AAG GAC CAG    192
Gln Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln
    50                  55                  60
CTC CAG TCC TAT ATC TGC TTC TGC CTC CCT GCC TTC GAG GGC CGG AAC    240
Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn
65                  70                  75                  80
TGT GAG ACG CAC AAG GAT GAC CAG CTG ATC TGT GTG AAC GAG AAC GGC    288
Cys Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly
                85                  90                  95
GGC TGT GAG CAG TAC TGC AGT GAC CAC ACG GGC ACC AAG CGC TCC TGT    336
Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys
            100                 105                 110
CGG TGC CAC GAG GGG TAC TCT CTG CTG GCA GAC GGG GTG TCC TGC ACA    384
Arg Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr
        115                 120                 125
CCC ACA GTT GAA TAT CCA TGT GGA AAA ATA CCT ATT CTA GAA AAA AGA    432
Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg
    130                 135                 140
AAT GCC AGC AAA CCC CAA GGC CGA ATT GTG GGG GGC AAG GTG TGC CCC    480
Asn Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Cys Pro
145                 150                 155                 160
AAA GGG GAG TGT CCA TGG CAG GTC CTG TTG TTG GTG AAT GGA GCT CAG    528
Lys Gly Glu Cys Pro Trp Gln Val Leu Leu Leu Val Asn Gly Ala Gln
                165                 170                 175
TTG TGT GGG GGG ACC CTG ATC AAC ACC ATC TGG GTG GTC TCC GCG GCC    576
Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala
            180                 185                 190
CAC TGT TTC GAC AAA ATC AAG AAC TGG AGG AAC CTG ATC GCG GTG CTG    624
His Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu
        195                 200                 205
GGC GAG CAC GAC CTC AGC GAG CAC GAC GGG GAT GAG CAG AGC CGG CGG    672
Gly Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg
    210                 215                 220
GTG GCG CAG GTC ATC ATC CCC AGC ACG TAC GAC AGG AAG ACT CTG AAC    720
Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Asp Arg Lys Thr Leu Asn
225                 230                 235                 240
CAC GAC ATC GCG CTG CTC CGC CTG CAC CAG CCC GTG GTC CTC ACT GAC    768
```

```
His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp
                245                 250                 255
CAT GTG GTG CCC CTC TGC CTG CCC GAA CGG ACG TTC TCT GAG AGG ACG   816
His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr
            260                 265                 270
CTG GCC TTC GTG CGC TTC TCA TTG GTC AGC GGC TGG GGC CAG CTG CTG   864
Leu Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu
        275                 280                 285
GAC CGT GGC GCC ACG GCC CTG GAG CTC ATG GTG CTC AAC GTG CCC CGG   912
Asp Arg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg
    290                 295                 300
CTG ATG ACC CAG GAC TGC CTG CAG CAG TCA CGG AAG GTG GGA GAC TCC   960
Leu Met Thr Gln Asp Cys Leu Gln Gln Ser Arg Lys Val Gly Asp Ser
305                 310                 315                 320
CCA AAT ATC ACG GAG TAC ATG TTC TGT GCC GGC TAC TCG GAT GGC AGC  1008
Pro Asn Ile Thr Glu Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser
                325                 330                 335
AAG GAC TCC TGC AAG GGG GAC AGT GGA GGC CCA CAT GCC ACC CAC TAC  1056
Lys Asp Ser Cys Lys Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr
            340                 345                 350
CGG GGC ACG TGG TAC CTG ACG GGC ATC GTC AGC TGG GGC CAG GGC TGC  1104
Arg Gly Thr Trp Tyr Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys
        355                 360                 365
GCA ACC GTG GGC CAC TTT GGG GTG TAC ACC AGG GTC TCC CAG TAC ATC  1152
Ala Thr Val Gly His Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile
    370                 375                 380
GAG TGG CTG CAA AAG CTC ATG CGC TCA GAG CCA CGC CCA GGA GTC CTC  1200
Glu Trp Leu Gln Lys Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu
385                 390                 395                 400
CTG CGA GCC CCA TTT CCC TAG                                     1221
Leu Arg Ala Pro Phe Pro

                405
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<210> 8
<211> 406
<212> PRT
<213> artificial sequence
<220>
<223> Amino acid sequence of recombinant mutant of blood coagulation factor VII in which the 5 amino acid residues from the 235th Valine to 239th threonine are replaced with Asp-Arg-Lys-Thr-Leu.
<400> 8

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Ala Asn Ala Phe Leu Glu Glu Leu Arg Pro Gly Ser Leu Glu Arg Glu
  1               5                  10                  15
Cys Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys
            20                  25                  30
Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp
        35                  40                  45
Gln Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln
    50                  55                  60
```

Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn
65 70 75 80
Cys Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly
85 90 95
Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys
100 105 110
Arg Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr
115 120 125
Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg
130 135 140
Asn Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Cys Pro
145 150 155 160
Lys Gly Glu Cys Pro Trp Gln Val Leu Leu Leu Val Asn Gly Ala Gln
165 170 175
Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala
180 185 190
His Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu
195 200 205
Gly Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg
210 215 220
Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Asp Arg Lys Thr Leu Asn
225 230 235 240
His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp
245 250 255
His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr
260 265 270
Leu Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu
275 280 285
Asp Arg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg
290 295 300
Leu Met Thr Gln Asp Cys Leu Gln Gln Ser Arg Lys Val Gly Asp Ser
305 310 315 320
Pro Asn Ile Thr Glu Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser
325 330 335
Lys Asp Ser Cys Lys Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr
340 345 350
Arg Gly Thr Trp Tyr Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys
355 360 365
Ala Thr Val Gly His Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile
370 375 380
Glu Trp Leu Gln Lys Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu
385 390 395 400
Leu Arg Ala Pro Phe Pro
405

<210> 9
<211> 1206
<212> DNA
<213> artificial sequence
<220>
<223> Amino acid sequence of recombinant mutant of blood coagulation fac

tor VII in which the 12 amino acid residues from the 311th leucine to 32
2th asparagine are replaced with Glu-Ala-Ser-Tyr-Pro-Gly-Lys, and cDNA se
quence coding thereof.

<400> 9

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GCC AAC GCG TTC CTG GAG GAG CTG CGG CCG GGC TCC CTG GAG AGG GAG     48
Ala Asn Ala Phe Leu Glu Glu Leu Arg Pro Gly Ser Leu Glu Arg Glu
 1               5                  10                  15
TGC AAG GAG GAG CAG TGC TCC TTC GAG GAG GCC CGG GAG ATC TTC AAG     96
Cys Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys
            20                  25                  30
GAC GCG GAG AGG ACG AAG CTG TTC TGG ATT TCT TAC AGT GAT GGG GAC    144
Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp
        35                  40                  45
CAG TGT GCC TCA AGT CCA TGC CAG AAT GGG GGC TCC TGC AAG GAC CAG    192
Gln Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln
    50                  55                  60
CTC CAG TCC TAT ATC TGC TTC TGC CTC CCT GCC TTC GAG GGC CGG AAC    240
Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn
65                  70                  75                  80
TGT GAG ACG CAC AAG GAT GAC CAG CTG ATC TGT GTG AAC GAG AAC GGC    288
Cys Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly
                85                  90                  95
GGC TGT GAG CAG TAC TGC AGT GAC CAC ACG GGC ACC AAG CGC TCC TGT    336
Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys
            100                 105                 110
CGG TGC CAC GAG GGG TAC TCT CTG CTG GCA GAC GGG GTG TCC TGC ACA    384
Arg Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr
        115                 120                 125
CCC ACA GTT GAA TAT CCA TGT GGA AAA ATA CCT ATT CTA GAA AAA AGA    432
Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg
    130                 135                 140
AAT GCC AGC AAA CCC CAA GGC CGA ATT GTG GGG GGC AAG GTG TGC CCC    480
Asn Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Cys Pro
145                 150                 155                 160
AAA GGG GAG TGT CCA TGG CAG GTC CTG TTG TTG GTG AAT GGA GCT CAG    528
Lys Gly Glu Cys Pro Trp Gln Val Leu Leu Leu Val Asn Gly Ala Gln
                165                 170                 175
TTG TGT GGG GGG ACC CTG ATC AAC ACC ATC TGG GTG GTC TCC GCG GCC    576
Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala
            180                 185                 190
CAC TGT TTC GAC AAA ATC AAG AAC TGG AGG AAC CTG ATC GCG GTG CTG    624
His Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu
        195                 200                 205
GGC GAG CAC GAC CTC AGC GAG CAC GAC GGG GAT GAG CAG AGC CGG CGG    672
Gly Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg
    210                 215                 220
GTG GCG CAG GTC ATC ATC CCC AGC ACG TAC GTC CCG GGC ACC ACC AAC    720
Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn
225                 230                 235                 240
CAC GAC ATC GCG CTG CTC CGC CTG CAC CAG CCC GTG GTC CTC ACT GAC    768
```

```
His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp
                245                 250                 255
CAT GTG GTG CCC CTC TGC CTG CCC GAA CGG ACG TTC TCT GAG AGG ACG    816
His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr
            260                 265                 270
CTG GCC TTC GTG CGC TTC TCA TTG GTC AGC GGC TGG GGC CAG CTG CTG    864
Leu Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu
        275                 280                 285
GAC CGT GGC GCC ACG GCC CTG GAG CTC ATG GTG CTC AAC GTG CCC CGG    912
Asp Arg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg
    290                 295                 300
CTG ATG ACC CAG GAC TGC GAA GCC TCC TAC CCT GGA AAG ATC ACG GAG    960
Leu Met Thr Gln Asp Cys Glu Ala Ser Tyr Pro Gly Lys Ile Thr Glu
305                 310                 315                 320
TAC ATG TTC TGT GCC GGC TAC TCG GAT GGC AGC AAG GAC TCC TGC AAG   1008
Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser Lys Asp Ser Cys Lys
                325                 330                 335
GGG GAC AGT GGA GGC CCA CAT GCC ACC CAC TAC CGG GGC ACG TGG TAC   1056
Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr Arg Gly Thr Trp Tyr
            340                 345                 350
CTG ACG GGC ATC GTC AGC TGG GGC CAG GGC TGC GCA ACC GTG GGC CAC   1104
Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys Ala Thr Val Gly His
        355                 360                 365
TTT GGG GTG TAC ACC AGG GTC TCC CAG TAC ATC GAG TGG CTG CAA AAG   1152
Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile Glu Trp Leu Gln Lys
    370                 375                 380
CTC ATG CGC TCA GAG CCA CGC CCA GGA GTC CTC CTG CGA GCC CCA TTT   1200
Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu Leu Arg Ala Pro Phe
385                 390                 395                 400
CCC TAG                                                          1206
Pro
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<210> 10
<211> 401
<212> PRT
<213> artificial sequence
<220>
<223> Amino acid sequence of recombinant mutant of blood coagulation factor Vll in which the 12 amino acid residues from the 311th leucine to 322th asparagine are replaced with Glu-Ala-Ser-Tyr-Pro-Gly-Lys.
<400> 10

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Ala Asn Ala Phe Leu Glu Glu Leu Arg Pro Gly Ser Leu Glu Arg Glu
  1               5                  10                  15
Cys Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys
            20                  25                  30
Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp
        35                  40                  45
Gln Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln
    50                  55                  60
Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn
65                  70                  75                  80
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Cys Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly
85 90 95
Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys
100 105 110
Arg Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr
115 120 125
Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg
130 135 140
Asn Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Cys Pro
145 150 155 160
Lys Gly Glu Cys Pro Trp Gln Val Leu Leu Leu Val Asn Gly Ala Gln
165 170 175
Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala
180 185 190
His Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu
195 200 205
Gly Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg
210 215 220
Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Val Pro Gly Thr Thr Asn
225 230 235 240
His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp
245 250 255
His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr
260 265 270
Leu Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu
275 280 285
Asp Arg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg
290 295 300
Leu Met Thr Gln Asp Cys Glu Ala Ser Tyr Pro Gly Lys Ile Thr Glu
305 310 315 320
Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser Lys Asp Ser Cys Lys
325 330 335
Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr Arg Gly Thr Trp Tyr
340 345 350
Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys Ala Thr Val Gly His
355 360 365
Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile Glu Trp Leu Gln Lys
370 375 380
Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu Leu Arg Ala Pro Phe
385 390 395 400
Pro

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<213> artificial sequence
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<223> Amino acid sequence of recombinant mutant of blood coagulation fac tor VII in which the 5 amino acid residues from the 235th Valine to 239t h threonine are replaced with Asp-Arg-Lys-Thr-Leu and the 12 amino acid residues from the 311th leucine to 322th asparagine are replaced with Gl

u-Ala-Ser-Tyr-Pro-Gly-Lys, and cDNA sequence coding thereof.

<400> 11

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GCC AAC GCG TTC CTG GAG GAG CTG CGG CCG GGC TCC CTG GAG AGG GAG     48
Ala Asn Ala Phe Leu Glu Glu Leu Arg Pro Gly Ser Leu Glu Arg Glu
 1               5                  10                  15
TGC AAG GAG GAG CAG TGC TCC TTC GAG GAG GCC CGG GAG ATC TTC AAG     96
Cys Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys
            20                  25                  30
GAC GCG GAG AGG ACG AAG CTG TTC TGG ATT TCT TAC AGT GAT GGG GAC    144
Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp
        35                  40                  45
CAG TGT GCC TCA AGT CCA TGC CAG AAT GGG GGC TCC TGC AAG GAC CAG    192
Gln Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln
    50                  55                  60
CTC CAG TCC TAT ATC TGC TTC TGC CTC CCT GCC TTC GAG GGC CGG AAC    240
Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn
65                  70                  75                  80
TGT GAG ACG CAC AAG GAT GAC CAG CTG ATC TGT GTG AAC GAG AAC GGC    288
Cys Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly
                85                  90                  95
GGC TGT GAG CAG TAC TGC AGT GAC CAC ACG GGC ACC AAG CGC TCC TGT    336
Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys
            100                 105                 110
CGG TGC CAC GAG GGG TAC TCT CTG CTG GCA GAC GGG GTG TCC TGC ACA    384
Arg Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr
        115                 120                 125
CCC ACA GTT GAA TAT CCA TGT GGA AAA ATA CCT ATT CTA GAA AAA AGA    432
Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg
    130                 135                 140
AAT GCC AGC AAA CCC CAA GGC CGA ATT GTG GGG GGC AAG GTG TGC CCC    480
Asn Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Cys Pro
145                 150                 155                 160
AAA GGG GAG TGT CCA TGG CAG GTC CTG TTG TTG GTG AAT GGA GCT CAG    528
Lys Gly Glu Cys Pro Trp Gln Val Leu Leu Leu Val Asn Gly Ala Gln
                165                 170                 175
TTG TGT GGG GGG ACC CTG ATC AAC ACC ATC TGG GTG GTC TCC GCG GCC    576
Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala
            180                 185                 190
CAC TGT TTC GAC AAA ATC AAG AAC TGG AGG AAC CTG ATC GCG GTG CTG    624
His Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu
        195                 200                 205
GGC GAG CAC GAC CTC AGC GAG CAC GAC GGG GAT GAG CAG AGC CGG CGG    672
Gly Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg
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Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Asp Arg Lys Thr Leu Asn
225                 230                 235                 240
CAC GAC ATC GCG CTG CTC CGC CTG CAC CAG CCC GTG GTC CTC ACT GAC    768
His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp
                245                 250                 255
```

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CAT GTG GTG CCC CTC TGC CTG CCC GAA CGG ACG TTC TCT GAG AGG ACG    816
His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr
            260                 265                 270
CTG GCC TTC GTG CGC TTC TCA TTG GTC AGC GGC TGG GGC CAG CTG CTG    864
Leu Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu
        275                 280                 285
GAC CGT GGC GCC ACG GCC CTG GAG CTC ATG GTG CTC AAC GTG CCC CGG    912
Asp Arg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg
    290                 295                 300
CTG ATG ACC CAG GAC TGC GAA GCC TCC TAC CCT GGA AAG ATC ACG GAG    960
Leu Met Thr Gln Asp Cys Glu Ala Ser Tyr Pro Gly Lys Ile Thr Glu
305                 310                 315                 320
TAC ATG TTC TGT GCC GGC TAC TCG GAT GGC AGC AAG GAC TCC TGC AAG   1008
Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser Lys Asp Ser Cys Lys
                325                 330                 335
GGG GAC AGT GGA GGC CCA CAT GCC ACC CAC TAC CGG GGC ACG TGG TAC   1056
Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr Arg Gly Thr Trp Tyr
            340                 345                 350
CTG ACG GGC ATC GTC AGC TGG GGC CAG GGC TGC GCA ACC GTG GGC CAC   1104
Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys Ala Thr Val Gly His
        355                 360                 365
TTT GGG GTG TAC ACC AGG GTC TCC CAG TAC ATC GAG TGG CTG CAA AAG   1152
Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile Glu Trp Leu Gln Lys
    370                 375                 380
CTC ATG CGC TCA GAG CCA CGC CCA GGA GTC CTC CTG CGA GCC CCA TTT   1200
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385                 390                 395                 400
CCC TAG                                                          1206
Pro
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<212> PRT
<213> artificial sequence
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<223> Amino acid sequence of recombinant mutant of blood coagulation factor VII in which the 5 amino acid residues from the 235th Valine to 239th threonine are replaced with Asp-Arg-Lys-Thr-Leu and the 12 amino acid residues from the 311th leucine to 322th asparagine are replaced with Glu-Ala-Ser-Tyr-Pro-Gly-Lys.
<400> 12

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Ala Asn Ala Phe Leu Glu Glu Leu Arg Pro Gly Ser Leu Glu Arg Glu
  1               5                  10                  15
Cys Lys Glu Glu Gln Cys Ser Phe Glu Glu Ala Arg Glu Ile Phe Lys
             20                  25                  30
Asp Ala Glu Arg Thr Lys Leu Phe Trp Ile Ser Tyr Ser Asp Gly Asp
         35                  40                  45
Gln Cys Ala Ser Ser Pro Cys Gln Asn Gly Gly Ser Cys Lys Asp Gln
     50                  55                  60
Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro Ala Phe Glu Gly Arg Asn
 65                  70                  75                  80
```

```
Cys Glu Thr His Lys Asp Asp Gln Leu Ile Cys Val Asn Glu Asn Gly
                85                  90                  95
Gly Cys Glu Gln Tyr Cys Ser Asp His Thr Gly Thr Lys Arg Ser Cys
            100                 105                 110
Arg Cys His Glu Gly Tyr Ser Leu Leu Ala Asp Gly Val Ser Cys Thr
        115                 120                 125
Pro Thr Val Glu Tyr Pro Cys Gly Lys Ile Pro Ile Leu Glu Lys Arg
    130                 135                 140
Asn Ala Ser Lys Pro Gln Gly Arg Ile Val Gly Gly Lys Val Cys Pro
145                 150                 155                 160
Lys Gly Glu Cys Pro Trp Gln Val Leu Leu Val Asn Gly Ala Gln
                165                 170                 175
Leu Cys Gly Gly Thr Leu Ile Asn Thr Ile Trp Val Val Ser Ala Ala
            180                 185                 190
His Cys Phe Asp Lys Ile Lys Asn Trp Arg Asn Leu Ile Ala Val Leu
        195                 200                 205
Gly Glu His Asp Leu Ser Glu His Asp Gly Asp Glu Gln Ser Arg Arg
    210                 215                 220
Val Ala Gln Val Ile Ile Pro Ser Thr Tyr Asp Arg Lys Thr Leu Asn
225                 230                 235                 240
His Asp Ile Ala Leu Leu Arg Leu His Gln Pro Val Val Leu Thr Asp
                245                 250                 255
His Val Val Pro Leu Cys Leu Pro Glu Arg Thr Phe Ser Glu Arg Thr
            260                 265                 270
Leu Ala Phe Val Arg Phe Ser Leu Val Ser Gly Trp Gly Gln Leu Leu
        275                 280                 285
Asp Arg Gly Ala Thr Ala Leu Glu Leu Met Val Leu Asn Val Pro Arg
    290                 295                 300
Leu Met Thr Gln Asp Cys Glu Ala Ser Tyr Pro Gly Lys Ile Thr Glu
305                 310                 315                 320
Tyr Met Phe Cys Ala Gly Tyr Ser Asp Gly Ser Lys Asp Ser Cys Lys
                325                 330                 335
Gly Asp Ser Gly Gly Pro His Ala Thr His Tyr Arg Gly Thr Trp Tyr
            340                 345                 350
Leu Thr Gly Ile Val Ser Trp Gly Gln Gly Cys Ala Thr Val Gly His
        355                 360                 365
Phe Gly Val Tyr Thr Arg Val Ser Gln Tyr Ile Glu Trp Leu Gln Lys
    370                 375                 380
Leu Met Arg Ser Glu Pro Arg Pro Gly Val Leu Leu Arg Ala Pro Phe
385                 390                 395                 400
Pro
```

【図面の簡単な説明】

【図１】　ＦＶＩＩの一次構造及び改変部位（星印）を示す図。

【図２】　ＦＶＩＩのプロテアーゼドメインアミノ酸配列を基にしたセリンプロテアーゼの基本構造を示す図。

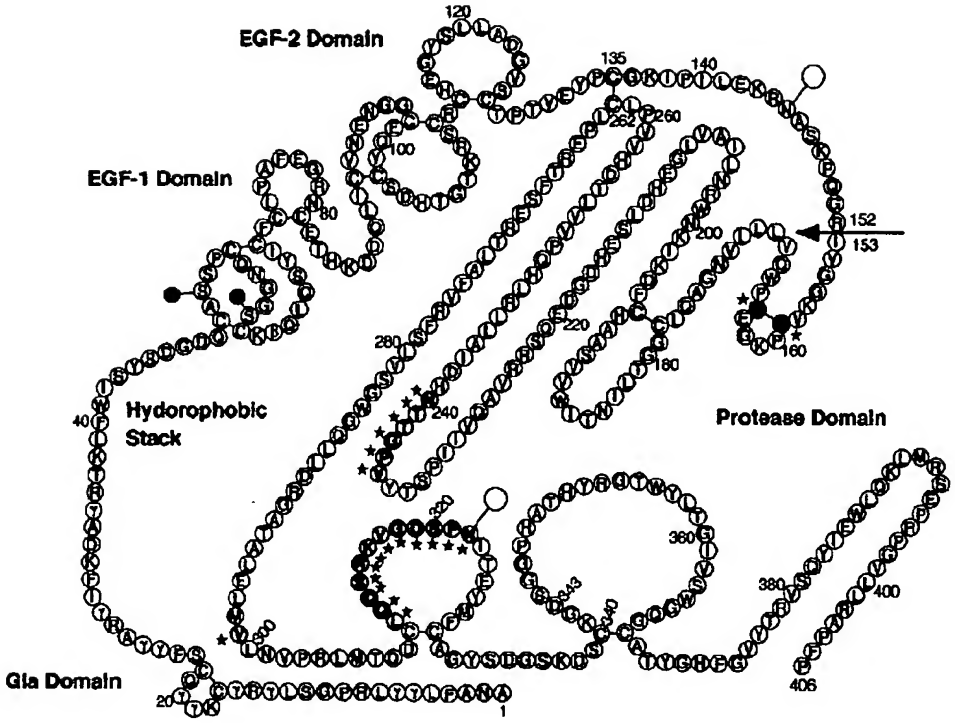
【図３】　Ｘ線立体構造既知の各種トリプシン族セリンプロテアーゼ間の３Ｄマルチアライメントを示す図。

【図４】　野生型ＦＶＩＩ（ＦＶＩＩ-Ｗｉｌｄ）及び各種ＦＶＩＩ改変体のアミノ酸配列の一部を示す図。本図はＦＶＩＩの１５３番目のイソロイシンよりＣ末側のアミノ酸配列のみ示したもので、１５２番目のアルギニンよりＮ末側のアミノ酸配列についてはいずれも改変は行っておらず野生型と同じである。

【図５】　ＦＶＩＩ改変体作製用プライマー配列を示す図。

【図６】　ＦＶＩＩ改変体発現ベクターの構築方法を示す図。

【図１】



【図２】

```
 I V G G K V C P K G E C P W Q V L L L V N G A Q L C G G T L I N T I W V V S A A
153                          βストランド1    βストランド2        βストランド3 192

 H C F D K I K N W R N L I A V L G E H D L S E H D G D E Q S R R V A Q V I I P S
193                 βストランド4                    βストランド5              232

 T Y V P G T T N H D I A L L R L H Q P V V L T D H V V P L C L P E R T F S E R T
233                 βストランド6                                              272

 L A F V R F S L V S G W G Q L L D R G A T A L E L M V L N V P R L M T Q D C L Q
273       βストランド7                          βストランド8                  312

 Q S R K V G D S P N I T E Y M F C A G Y S D G S K D S C K G D S G G P H A T H Y
313                          βストランド9                    βストランド10 352

 R G T W Y L T G I V S W G Q G C A T V G H F G V Y T R V S Q Y I E W L Q K L M R
353    βストランド11                        βストランド12                    392

 S E P R P G V L L R A P F P
393
```

【図３】

sequence 1: ヒト血液凝固第VII因子 (pdb ID 1DAN)
sequence 2: ヒトトリプシン (pdb ID 1TRN)
sequence 3: ブタ血液凝固第IX因子 (pdb ID 1PFX)
sequence 4: ウシトリプシン (pdb ID 1TLD)
sequence 5: ヒト血液凝固第X因子 (pdb ID 1HCG)
sequence 6: ヒトプロテインC (pdb ID 1AUT)
sequence 7: ブタカリクレインA (pdb ID NPKA)
sequence 8: ウシキモトリプシン (pdb ID 5CHA)
sequence 9: ブタエラスターゼ (pdb ID 3EST)
sequence10: ヒトαトロンビン (pdb ID 1PPB)
sequence11: ヒト多形核白血球プロテアーゼ3 (pdb ID 1FUJ)
sequence12: ラットトニン (pdb ID 1TON)
sequence13: ヒト好中球エラスターゼ (pdb ID 1HNE)
sequence14: ヒトウロキナーゼ型プラスミノーゲンアクチベータ (pdb ID 1LMW)
sequence15: ヒトカテプシンG (pdb ID 1CGH)
sequence16: ラット肥満細胞プロテアーゼ (pdb ID 3RP2)
sequence17: ヒト組織型プラスミノーゲンアクチベータ (pdb ID 1RTF)

(図中@位置はすべてのプロテアーゼでのCα位置が1Å以内で保存されている構造保存部位を示す)

	βストランド5-βストランド6近傍のアライメント	βストランド8-βストランド9近傍のアライメント
	@@@@@@@@@@:@@@:::::::::@@@@@@@@@@@@	@@@@@@@@@@@@@:::::::::::::::::::@@@@@@@
sequence 1:	SRRVAQVIIPSTYVP----G-TTNHDIALLRLHQ	ALELMVLNVPRLMTQDCLQQSRKVGDSPNITEYMFCAG
sequence 2:	FINAAKIIRHPQYDR----K-TLNNDIMLIKLSS	PDELQCLDAPVLSQAKCEA-S-Y---PGKITSNMFCVG
sequence 3:	RRNVIRAIPHHSYNAT---VNKYSHDIALLELDE	ATILQYLKVPLVDRATCL--R-ST--KFTIYSNMFCAG
sequence 4:	FISASKSIVHPSYNS----N-TLNNDIMLIKLKS	PDVLKCLKAPILSDSSCKS-A-Y---PGQITSNMFCAG
sequence 5:	VHEVEVVIKHNRFTK----E-TYDFDIAVLRLKT	STRLKMLEVPYVDRNSCKL-S-S---SFIITQNMFCAG
sequence 6:	DLDIKEVFVHPNYSK----S-TTDNDIALLHLAQ	TFVLNFIKIPVVPHNECSE-V-M---SNMVSENMLCAG
sequence 7:	FFGVTADFPHPGFNLSA-DGKDYSHDLMLLRLQS	PDEIQCVQLTLLQNTFCA--D-AH--PDEVTESMLCAG
sequence 8:	KLKIAKVFKNSKYNS----L-TINNDITLLKLST	PDRLQQASLPLLSNTNCKK-Y-W---GTKIKDAMICAG
sequence 9:	YVGVQKIVVHPYWNT--D-DVAAGYDIALLRLAQ	AQTLQQAYLPTVDYAICSS-SSYW--GSTVKNSMVCAG
sequence10:	ISMLEKIYIHPRYNW----RENLDRDIALMKLKK	PSVLQVVNLPIVERPVCKD-S-T---RIRITDNMFCAG
sequence11:	HFSVAQVFLN-KYDA----E-NKLNDILLIQLSS	AQVLQELNVTVVT--FFC----------R-PHNICTF
sequence12:	RRLVRQSFRHPDYIP--LPVHDHSNDLMLLHLSE	SHDLQCVNIHLLSNEKCI--E-TY--KDNVTDVMLCAG
sequence13:	VFAVQRIFED-GYDP----V-NLLNDIVILQLNG	ASVLQELNVTVVT--SLC----------R-RSNVCTL
sequence14:	KFEVENLILHKDYSA--D-TLAHHNDIALLKIRS	PEQLKMTVVKLISHRECQQPH-YY--GSEVTTKMLCAA
sequence15:	HITARRAIRHPQYNQ----R-TIQNDIMLLQLSR	TDTLREVQLRVQRDRQCLR-I-F---GSYDPRRQICVG
sequence16:	KIKVEKQIIHESYNS----V-PNLHDIMLLKLEK	SYTLREVELRIMDEKACVD-Y-R---Y-YEYKFQVCVG
sequence17:	KFEVEKYIVHKEFDD----D-TYDNDIALLQLKS	SERLKEAHVRLYPSSRCTSQH-LL--NRTVTDNMLCAG

【図４】

VII-Wild
IVGGKVCPKGECPWQVLLLVNGAQLCGGTLINTIWVVSAAHCFDKIKNWRNLIAVLGEHD
LSEHDGDEQSRRVAQVIIPSTYVPGTTNHDIALLRLHQPVVLTDHVVPLCLPERTFSERT
LAFVRFSLVSGWGQLLDRGATALELMVLNVPRLMTQDCLQQSRKVGDSPNITEYMFCAGY
SDGSKDSCKGDSGGPHATHYRGTWYLTGIVSWGQGCATVGHFGVYTRVSQYIEWLQKLMR
SEPRPGVLLRAPFP

VII-5
IVGGKVAPKGEAPWQVLLLVNGAQLCGGTLINTIWVVSAAHCFDKIKNWRNLIAVLGEHD
LSEHDGDEQSRRVAQVIIPSTYVPGTTNHDIALLRLHQPVVLTDHVVPLCLPERTFSERT
LAFVRFSLVSGWGQLLDRGATALELMVLNVPRLMTQDCLQQSRKVGDSPNITEYMFCAGY
SDGSKDSCKGDSGGPHATHYRGTWYLTGIVSWGQGCATVGHFGVYTRVSQYIEWLQKLMR
SEPRPGVLLRAPFP

VII-6
IVGGKVCPKGEAPWQVLLLVNGAQLCGGTLINTIWVVSAAHCFDKIKNWRNLIAVLGEHD
LSEHDGDEQSRRVAQVIIPSTYVPGTTNHDIALLRLHQPVVLTDHVVPLCLPERTFSERT
LAFVRFSLVSGWGQLLDRGATALELMCLNVPRLMTQDCLQQSRKVGDSPNITEYMFCAGY
SDGSKDSCKGDSGGPHATHYRGTWYLTGIVSWGQGCATVGHFGVYTRVSQYIEWLQKLMR
SEPRPGVLLRAPFP

VII-30
IVGGKVCPKGECPWQVLLLVNGAQLCGGTLINTIWVVSAAHCFDKIKNWRNLIAVLGEHD
LSEHDGDEQSRRVAQVIIPSTYDRKTLNHDIALLRLHQPVVLTDHVVPLCLPERTFSERT
LAFVRFSLVSGWGQLLDRGATALELMVLNVPRLMTQDCLQQSRKVGDSPNITEYMFCAGY
SDGSKDSCKGDSGGPHATHYRGTWYLTGIVSWGQGCATVGHFGVYTRVSQYIEWLQKLMR
SEPRPGVLLRAPFP

VII-31
IVGGKVCPKGECPWQVLLLVNGAQLCGGTLINTIWVVSAAHCFDKIKNWRNLIAVLGEHD
LSEHDGDEQSRRVAQVIIPSTYVPGTTNHDIALLRLHQPVVLTDHVVPLCLPERTFSERT
LAFVRFSLVSGWGQLLDRGATALELMVLNVPRLMTQDCEASYP-----GKITEYMFCAGY
SDGSKDSCKGDSGGPHATHYRGTWYLTGIVSWGQGCATVGHFGVYTRVSQYIEWLQKLMR
SEPRPGVLLRAPFP

VII-39
IVGGKVCPKGECPWQVLLLVNGAQLCGGTLINTIWVVSAAHCFDKIKNWRNLIAVLGEHD
LSEHDGDEQSRRVAQVIIPSTYDRKTLNHDIALLRLHQPVVLTDHVVPLCLPERTFSERT
LAFVRFSLVSGWGQLLDRGATALELMVLNVPRLMTQDCEASYP-----GKITEYMFCAGY
SDGSKDSCKGDSGGPHATHYRGTWYLTGIVSWGQGCATVGHFGVYTRVSQYIEWLQKLMR
SEPRPGVLLRAPFP

（下線部は改変部位を表す）

【図５】

①VII-PWN Sense；5′-GGGGTCGACATGGTCTCCCAGGCCCTCAGGCTCCTCTGCCTTCTG-3′
Factor VII Wild type のシグナル配列からのプライマーデザイン
5′-GGGGTCGACATGGTCTCCCAGGCCCTCAGGCTCCTCTGCCTTCTG-3′
SalI M V S Q A L R L L C L L

②VII-PWC AntiS；5′-CCCGGATCCCTAGGGAAATGGGGCTCGCAGGAGGACTCCTGGGCG-3′
Factor VII Wild type のカルボキシ末端までのプライマーデザイン
5′-CCCGGATCCCTAGGGAAATGGGGCTCGCAGGAGGACTCCTGGGCG-3′
BamHI

③VII-P5-1 Sense；5′-ATTGTGGGGGGCAAGGTGGCCCCCAAAGGGGAGGCCCCATGGCAGGTC-3′
④VII-P5-2 AntiS；5′-GACCTGCCATGGGGCCTCCCCTTTGGGGGCCACCTTGCCCCCCACAAT-3′
VII-5のプライマーデザイン（C159A, C164A）
5′-ATTGTGGGGGGCAAGGTGGCCCCCAAAGGGGAGGCCCCATGGCAGGTC-3′
3′-TAACACCCCCCGTTCCACCGGGGGTTTCCCCTCCGGGGTACCGTCCAG-5′
I V G G K V A P K G E A P W Q V

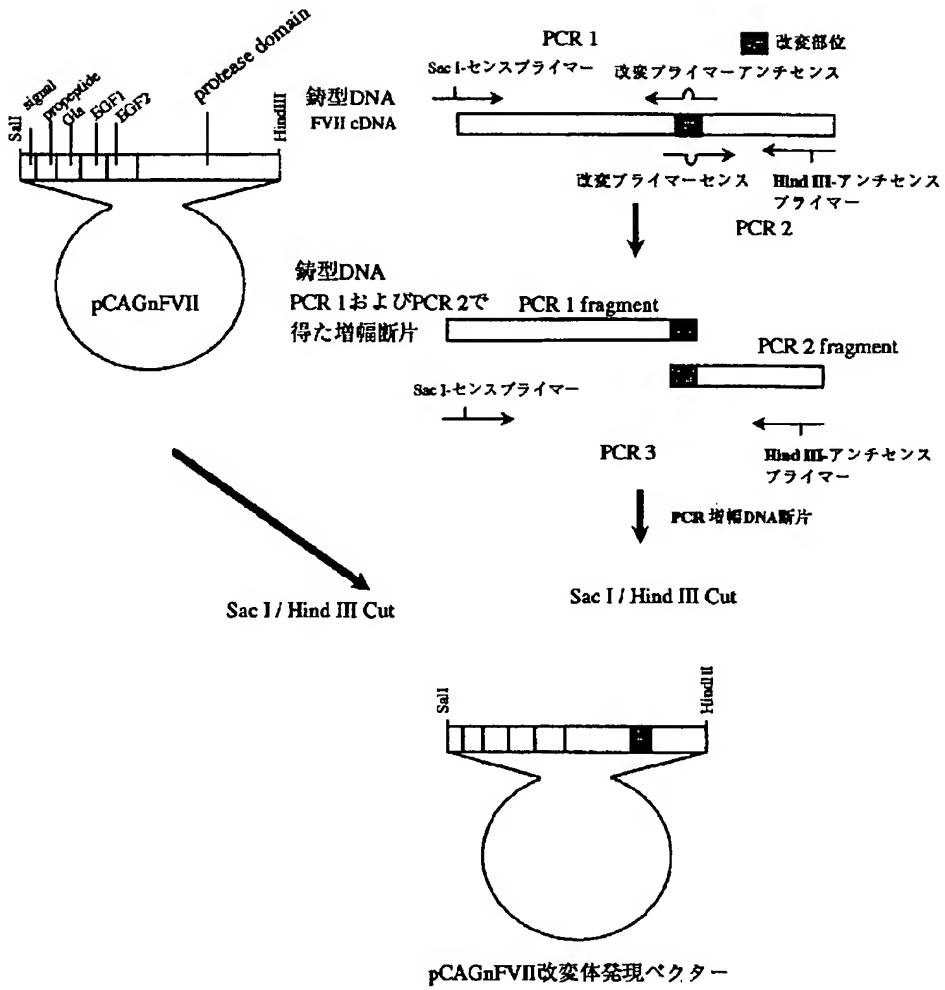
⑤VII-P6-1 Sense；5′-TGCCCCAAAGGGGAGGCCCCATGGCAGGTC-3′
⑥VII-P6-2 AntiS；5′-GACCTGCCATGGGGCCTCCCCTTTGGGGCA-3′
VII-6のプライマーデザイン①（C164A）
5′-TGCCCCAAAGGGGAGGCCCCATGGCAGGTC-3′
3′-ACGGGGTTTCCCCTCCGGGGTACCGTCCAG-5′
C P K G E A P W Q V

⑦VII-P6-3 Sense；5′-CTGGAGCTCATGTGCCTCAACGTGCCCCGG-3′
⑧VII-P6-4 AntiS；5′-CCGGGGCACGTTGAGGCACATGAGCTCCAG-3′
VII-6のプライマーデザイン②（V299C）
5′-CTGGAGCTCATGTGCCTCAACGTGCCCCGG-3′
3′-GACCTCGAGTACACGGAGTTGCACGGGGCC-5′
L E L M C L N V P R

⑨VII-P30-1 Sense；5′-ATCCCCAGCACGTACGACAGGAAGACTCTGAACCACGACATCGCGCTG-3′
⑩VII-P30-2 AntiS；5′-CAGCGCGATGTCGTGGTTCAGAGTCTTCCTGTCGTACGTGCTGGGGAT-3′
VII-30のプライマーデザイン（VPGTTN→DRKTLN）
5′-ATCCCCAGCACGTACGACAGGAAGACTCTGAACCACGACATCGCGCTG-3′
3′-TAGGGGTCGTGCATGCTGTCCTTCTGAGACTTGGTGCTGTAGCGCGAC-5′
I P S T Y D R K T L N H D I A L

⑪VII-P31-1 Sense；5′-ATGACCCAGGACTGCGAAGCCTCCTACCCTGGAAAGATCACGGAGTACATG-3′
⑫VII-P31-2 AntiS；5′-CATGTACTCCGTGATCTTTCCAGGGTAGGAGGCTTCGCAGTCCTGGGTCAT-3′
VII-31のプライマーデザイン（LQQSRKVGDSPN→EASYPGK）
5′-ATGACCCAGGACTGCGAAGCCTCCTACCCTGGAAAGATCACGGAGTACATG-3′
3′-TACTGGGTCCTGACGCTTCGGAGGATGGGACCTTTCTAGTGCCTCATGTAC-5′
M T Q D C E A S Y P G K I T E Y M

【図６】



フロントページの続き

(72)発明者　岩永　貞昭
福岡県福岡市東区水谷２-34-16

Ｆターム(参考)　4B024 AA01 BA11 CA04 DA02 DA06
EA04 GA11 GA13 GA18 HA01
HA04 HA06
4B050 CC04 CC06 DD11 FF14E
HH01 LL01
4C084 AA02 AA07 BA01 BA08 BA22
CA18 CA53 DC14 NA05 ZA532
ZC542
4H045 AA10 AA30 BA10 BA12 BA13
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